
THE UNIVERSITY
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# The Role of Severe Obesity in Osteoarthritis

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## Declaration

I hereby declare that the work presented in this thesis was carried out entirely by me or otherwise as stated below:

Dr Robert Wallace calculated Bone Mineral Density values for samples analysed in Chapter 7.

The thesis has been composed by me and is submitted for the fulfilment of the degree of Doctor of Philosophy, and not for any other degree or qualification.

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June 2016

# Abstract

Osteoarthritis (OA) is the most common degenerative joint disease affecting more than 40% of people above the age of 65 (Neogi et al., 2013). Obesity is one of the main risk factors of OA and has become a major problem in Western societies. With sedentary lifestyle and the aging of the population, it is estimated that more than 50% of British adults will be obese in 2030 (Wang et al., 2011). So far, the effect of obesity on joint degeneration has primarily been explained by the increased load on the joints. However, a growing number of studies have revealed that adipose tissue can affect cartilage and other joint tissues at a molecular level. The main goal of this thesis was to investigate the role of local knee joint tissues in obese patients with OA.

The expression of molecular markers was investigated in local knee tissues: cartilage, synovium, infrapatellar fat pad (IPFP) and subchondral bone collected during Total Knee Replacement (TKR). A range of techniques (RT-PCR, Real Time qPCR, WB, IHC/ICC and ELISA) was used to examine the differences between genes and proteins expression in both lean and obese patients with OA. Further, the local immune cell infiltration was investigated in knee adipose tissue depots (synovium and IPFP) using flow cytometry. In addition, the subchondral bone microstructure was analysed using micro-Computed Tomography (μCT) and IHC techniques.

Chondrocytes from OA patients were found to express a range of obesity-related genes. ADIPOR1 was produced significantly higher than ADIPOR2 in OA chondrocytes. Furthermore, CCL2 was produced at higher while PPARγ and visfatin were produced at a lower level in obese patients' chondrocytes in comparison to lean ones. Synovium and IPFP also expressed a range of obesity-related genes. PPARγ and visfatin expression was lower in obese synovium and IPFP in comparison to lean. Surprisingly, adiponectin was expressed at a significantly lower level in obese patients' synovium. In contrast, adiponectin was not differently expressed in lean and obese patients' IPFP. The IPFP was found to be a significantly higher producer of PPARγ and adiponectin in comparison to synovium. Synovium, on the other hand,

has an increased expression of VCAM-1, TLR4 and CCL2 in obese patients. An increased number of macrophages (defined by CD45+CD14+ and CD14+CD206+ markers expression) was detected in the synovium and IPFP from obese OA patients. Furthermore, there was an increased number of CD86+CD14+ cells in the synovium from obese patients. Other macrophage-related proteins including HLA-DR, CD36 were also expressed at a higher level in synovium from obese patients. T-lymphocyte detection revealed a higher number of CD3+CD4+ T cells in the synovium (but not IPFP) from obese patients but no change in the CD3+CD8+ population in both the synovium and IPFP. Subchondral bone analysis revealed possible differences in this tissue in obese male patients with OA in comparison to lean patients. μCT examination of subchondral bone showed a significantly lower bone mineral density (BMD) in obese in comparison to lean male OA patients. IHC analysis of bone sections suggested that there was an increased number of bone marrow adipose tissue macrophages. In addition, osteoblasts obtained from obese OA donors expressed a significantly higher level of ADIPOR2 and lower level of PPARγ mRNA in comparison to lean patients' osteoblasts.

The data obtained suggests that there were differences between lean and obese patients with OA at a molecular level. This proposes possible future directions for targeting these diseases. The limitation of the study were as follows: 1) possible different stages of end-stage OA between analysed patients, which could lead to differences in obtained data, 2) no non-OA control samples included in the study. However, the presented study may suggest that all tissues in the knee joint contribute to the interplay between OA and obesity. In addition, the data obtained is the first to suggest that there are differences in gene and protein expression in the synovium and IPFP from the same donor. Furthermore, there are differences in the immune cell populations in local adipose tissue depots (synovium and IPFP) from OA joints, which are linked to obesity. All of this data has helped to increase our understanding of the interaction between obesity and OA.

# Lay Summary

Osteoarthritis (OA) is the most common degenerative joint disease affecting more than 40% of people above the age of 65 (Neogi et al., 2013). Obesity is one of the main risk factors for OA and has become a major problem in Western societies. With sedentary lifestyle and the aging of the population, it is estimated that more than 50% of British adults will be obese in 2030 (Wang et al., 2011). So far, the effect of obesity on OA has primarily been explained by the increased load on the joints. However, a growing number of studies have revealed that adipose tissue can affect cartilage and other joint tissues at the molecular level. Thus, the main goal of this thesis was to investigate the behaviour of local knee joint tissues in obese patients with OA.

The expression of molecular markers was investigated in local knee tissues: cartilage, synovium, infrapatellar fat pad (IPFP) and subchondral bone collected during Total Knee Replacement (TKR). A range of molecular biology techniques was used to examine differences between genes and protein expression between lean and obese patients with OA. In addition, the immune cell infiltration in the adipose tissue depots (synovium and IPFP) was investigated and the microstructure of the subchondral bone was analysed.

The data obtained suggested that cells from cartilage and bone (chondrocytes and osteoblasts) in addition to local tissues, surrounding the knee joint in OA patients, expressed a range of obesity-related genes and proteins. Obese patients' tissues expressed certain markers at higher levels including proteins, which stimulate cell migration such as CCL2 and other markers at lower levels including visfatin and PPARγ.

Local knee fatty tissues from obese patients were found to have an increased number of immune cells, which could facilitate OA progression. The bone microstructure was also analysed in samples from lean and obese OA patients and revealed that despite the increased weight there was a decrease in bone mineral density in obese

male subjects with OA in addition to an increased number of immune cells within the bone marrow.

The data obtained suggest that there were differences between lean and obese patients with OA at a molecular level, which could be suitable targets for drug modifying agents for these diseases. Furthermore, the findings of this thesis suggest that all tissues building the knee joint could be potent agents in the OA and obesity pathogenesis. In addition, the data obtained suggest for the first time that there are differences in gene and protein expression in the synovium and IPFP from the same donor. Moreover, there were differences in the immune cells population in local adipose tissue depots (synovium and IPFP) connected with obesity in OA joints. All of this data has helped to increase our understanding of the interaction between obesity and OA.

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I dedicate this thesis to my Parents and the whole family. I am forever grateful for your support, love and faith in me during my education. Without your strength and pride, I wouldn't be in this place. *Serdecznie dziękuję Tobie Mamo i Tato za wasze wsparcie, miłość i wiarę we mnie przez całe życie.*

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# Abbreviation

**ADAMTS** – A Disintegrin And Metalloproteinase with Thrombospondin Motifs

**ADIPOQ**- Adiponectin

**ADIPOR1**- Adiponectin Receptor 1

**ADIPOR2** - Adiponectin Receptor 2

**ALP** – Alkaline Phosphatase

**ATM** – Adipose Tissue Macrophages

**B2M** – β2 microglobulin

**BMD** – Bone Mineral Density

**BMI** – Body Mass Index

**BV/TV**- Bone Volume/Total Volume

**CD** – Cluster of Differentiation

**µCT** – micro Computed Tomography

**CCL2** – Chemokine (C-C) motif Ligand 2

**C/EBPβ** - CCAAT/Enhancer Binding Protein β

**CMKLR1** - Chemokine Like Receptor 1

**DAMP** - Damage-Associated Molecular Pattern

**DNA** - Deoxyribonucleic Acid

**ECM** – Extracellular Matrix

**FFA** – Free Fatty Acids

**FLS** – Fibroblast-Like Synoviocytes

**FTO** – Fat Mass and Obesity Associated Gene

**GAG -** Glycosaminoglycan

**H&E** – Hematoxylin & Eosin

**IF** – Immunofluorescence

**IFN -** Interferon

**IHC** – Immunohistochemistry

**IL** – Interleukin

**IPFP** – Infrapatellar Fat Pad

**LCN2** – Lipocalin 2

**LEP** – Leptin

**LEPR** – Leptin Receptor

**LPS** – Lipopolysaccharide

**MCP-1** – Monocyte Chemoattractant Protein-1

**MMP** – Matrix Metalloproteinase

**MSC** – Mesenchymal Stem Cells

**NAMPT** – Nicotinamide Phosphoribosyltransferase

**OA** – Osteoarthritis

**OPG** – Osteoprotegrin

**PBEF** – pre -B-cell Colony-Enhancing Factor 1

**PCR** – Polymerase Chain Reaction

**PGE** – Prostaglandin E

**PPAR** – Peroxisome Proliferator-Activated Receptor

**PRR** – Pattern Recognition Receptor

**RA** – Rheumatoid Arthritis

**RNA** – Ribonucleic Acid

**SAT** – Subcutaneous Adipose Tissue

**SF** – Synovial Fluid

**SVF** – Stromovascular Fraction

**T2DM**- Type 2 Diabetes Mellitus

**TGF** – Transforming Growth Factor

**TIG2** – Tazarotene-Induced Gene 2

**TLR** – Toll-Like Receptor

**TNC** – Tenascin-C

**TNF** – Tumor Necrosis Factor

**Tr.N.** – Trabecular Number

**Tr.Sp.** – Trabecular Spacing

**Tr.Th.** – Trabecular Thickness

**TZD -** Thiazolidinediones

**VAT** – Visceral Adipose Tissue

**VCAM-1** – Vascular Cell Adhesion Molecule1

**WAT** – White Adipose Tissue

# Chapter 1: Literature Review

## 1.1 Introduction

Osteoarthritis (OA) is the most common type of arthritis among UK citizens. Every year, more than 140,000 hip and knee replacements are performed in order to reduce pain and improve living conditions (*from Arthritis UK*). OA is a degenerative joint disease leading to disability. Currently, joint replacement is the most common treatment for end-stage disease and the most effective way to eliminate pain.

Obesity is a steadily increasing problem in developed countries and is considered to be a strong risk factor for OA (Silverwood et al., 2015). Although there are millions of people affected by OA, the detailed mechanism and precise cellular and molecular details have only been partially elucidated. The main goal of this project was to analyse further the molecular details of OA between patients undergoing knee replacement surgery with attention to the role of obesity in OA pathogenesis/progression. The main focus was on four different tissues, which make up the knee joint, namely: cartilage, synovial tissue, knee fat pad (so called infrapatellar fat pad (IPFP)), and bone.

## 1.2 Osteoarthritis

### 1.2.1 Cartilage composition

Cartilage is a connective tissue, which forms the articulating surface of synovial joints. Its unique composition is important in maintaining the joint structure and function. Human knee joint articular cartilage is 2-4 mm thick. It is an avascular tissue, which does not possess a lymphatic supply. It, therefore, relies on adjacent tissues including synovium or subchondral bone to provide essential nutrients as well as remove metabolic products.

Proteoglycans and collagens are macromolecules produced by cartilage. They are assembled in a specific order forming four layers that allow cartilage tissue to maintain frictionless movement of the whole joint. Aggrecan which is the major proteoglycan in the cartilage together with small glycoprotein called link protein binds to a long chain of hyaluronic acid (HA) and form multimolecular structures called proteoglycan aggregates. The negative charge of proteoglycans aggregates enables cartilage to maintain high water content (up to 75%). The specific structure of collagens and proteoglycans enables cartilage to respond to the compressive forces which it experiences (Silverwood et al., 2015).

In normal, healthy cartilage, there is a balance between processes responsible for matrix synthesis and those responsible for degradation. Chondrocytes within articular cartilage are the exclusive source of extracellular matrix (ECM). These dynamic changes help in sustaining the protective role of cartilage in weight-bearing. Cartilage is strong, flexible and semi-rigid. There are three types of cartilage: hyaline, fibrocartilage and elastic. The most common is hyaline cartilage, the main constituent of synovial joints including knees and hips. Fibrocartilage can be found in tendons and in vertebral discs while elastic cartilage is found in the external ear. There are two main cell types found in cartilage: immature/progenitor chondroblast that divide, proliferate, produce and secrete all ECM components once they are trapped in the ECM and become chondrocytes which reside in lacunae and maintain production of ECM.

Healthy articular cartilage can be divided into various zones (see Figure 1.1). The Superficial/Tangential zone (STZ), represents 10-20% of the total cartilage volume and protects the structure from shear stresses. Collagen fibres within this zone are tightly packed and in parallel alignment. Their shape is flat and they are specialised to produce collagen II and lubricin which helps in lubrication of the joint surfaces. Proteoglycan production is low in this zone. Below the STZ there is the middle zone (MZ) which represents 40-60% of total cartilage volume. Collagen fibrils in the MZ are thicker and organised perpendicularly, while chondrocytes are fewer and more spherical. The middle zone is composed mainly of proteoglycans. The role of MZ is to provide resistance to compressive forces. The Deep Zone (DZ) representing 30-40% of total cartilage, in which collagen fibrils are arranged vertically, distributes loads and also provides resistance to compressive forces. It contains the thickest collagen fibrils, the highest proteoglycan content, and lowest water concentration. Below the deep zone, there is a calcified cartilage distinguished from the DZ by the tidemark. The tidemark is a basophilic line, which designates the boundary between calcified and uncalcified cartilage. Calcified cartilage separates the uncalcified cartilage from the subchondral bone (Sophia Fox et al., 2009).

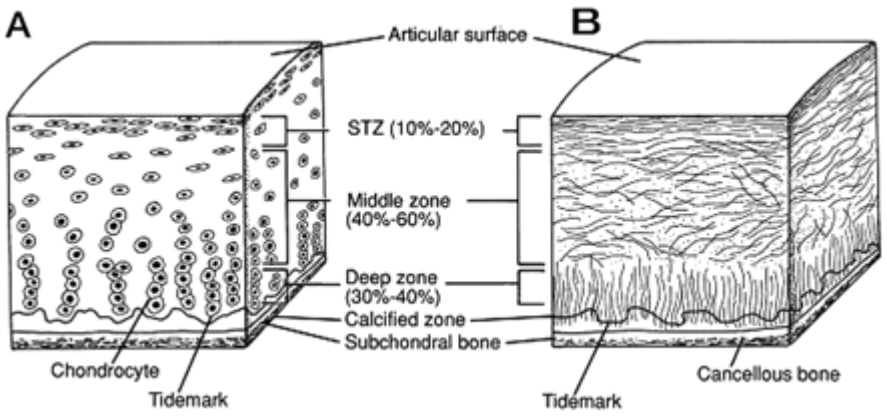



**Figure 1. 1 The structure and composition of articular cartilage.**
Diagram taken from (Sophia Fox et al., 2009). Main cartilage zones are presented. STZ – superficial/tangential zone.

### 1.2.2 Knee Joint

The knee joint is the largest joint in the human body (Kulowski, 2007). It consists of three articulations: two tibiofemoral compartments between the tibia and femur and one patellofemoral articulation between the patella and femur. The knee joint is a synovial, hinge joint, which allows flexion, extension and slight internal and external rotation. The tibiofemoral compartments include the capsule, several types of ligaments and the menisci (see Figure 1.2).

The articular joint capsule is an envelope surrounding the synovial joint. It consists of an outer fibrous tissue and an inner synovial layer. The menisci (medial and lateral) are crescent-shaped articular discs that partly divide the joint space between the tibia and femur. They comprise connective tissue rich in collagen fibers and chondrocyte-like cells. The main role of the menisci is to increase the area of contact between the two bone surfaces. Therefore, they decrease compressive stress to help with lubrication of articular cartilage, in addition to shock absorption. Ligaments composed of fibrous connective tissue connect bones to other bones. In the knee joint, they provide stability by limiting certain movements of the joint.

The main role of patellofemoral ligaments is to prevent displacement of the patella. The infrapatellar fat pad is the largest fatty tissue depot in the joint, it lies posterior to the patella ligament.

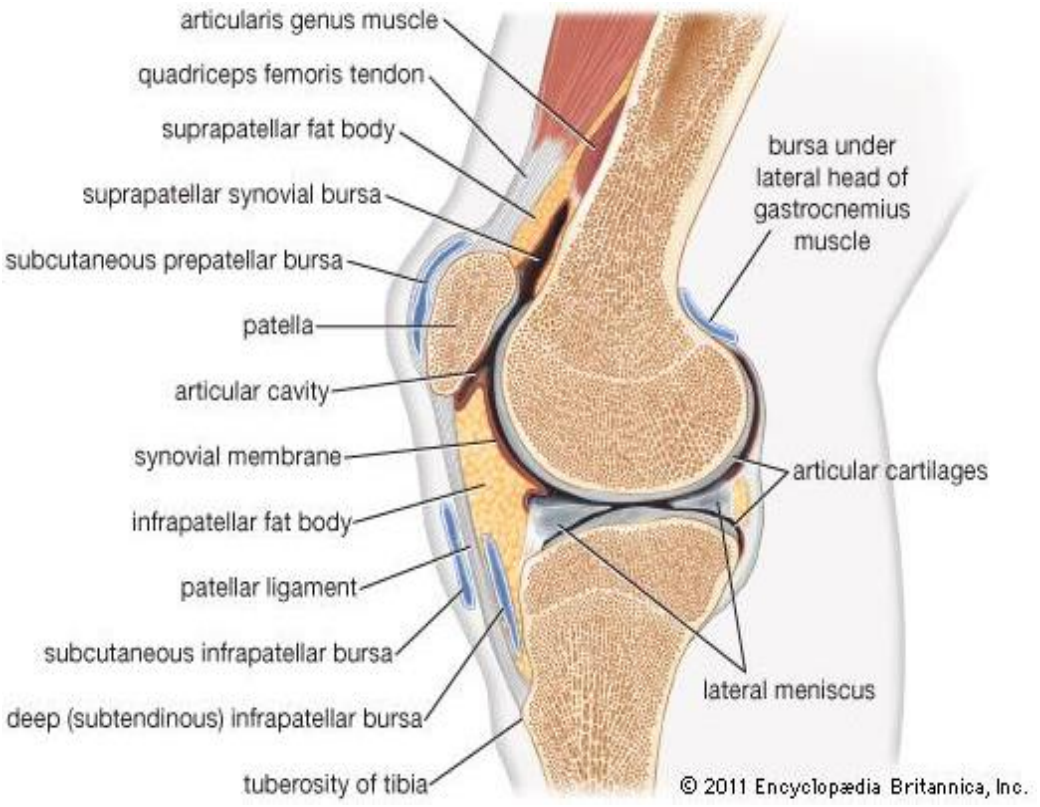



**Figure 1. 2 Knee parasagittal section-lateral to the midline of the knee.**
The main components of the knee are presented. Image adapted from *Encyclopedia Britannica inc*.

### 1.2.3 Cartilage in OA

When the structure of healthy cartilage is disrupted, its protective role is lost. The damage can result from previous joint injury, but is also strongly correlated with aging, gender, and obesity. Though the aetiology of OA is not fully understood, genetic, environmental, mechanical and metabolic factors are considered to play an important role. OA is a multifactorial disease affecting not only the cartilage but also other tissues of the joint including bone, fat pad and synovium, which are all involved in the further progression of OA. Currently, there are no satisfactory treatments for OA. Available drugs only relieve symptoms of OA rather than modifying progression of the disease.

Degeneration of cartilage begins with overproduction of aggrecanases, members of A Disintegrin, a Metalloproteinase with Thrombospondin Motifs (ADAMTS) family (Bondeson et al., 2008). Aggrecanases are proteases responsible for degrading

aggrecan. These events result in stopping the water intake and is followed by an overproduction of matrix metalloproteinases (MMPs) degrading collagen and other cartilage components as well as cysteine proteinases (Milner et al., 2006). Overproduction of these degrading proteins leads to a predominance of catabolic processes and an inevitable loss of cartilage. Destruction of aggrecan and collagen II leads to the loss of proper function of cartilage and is believed to be the first step in the onset of OA (Rousseau et al., 2007). Representative Cartilage degeneration showed in Figure 1.3.

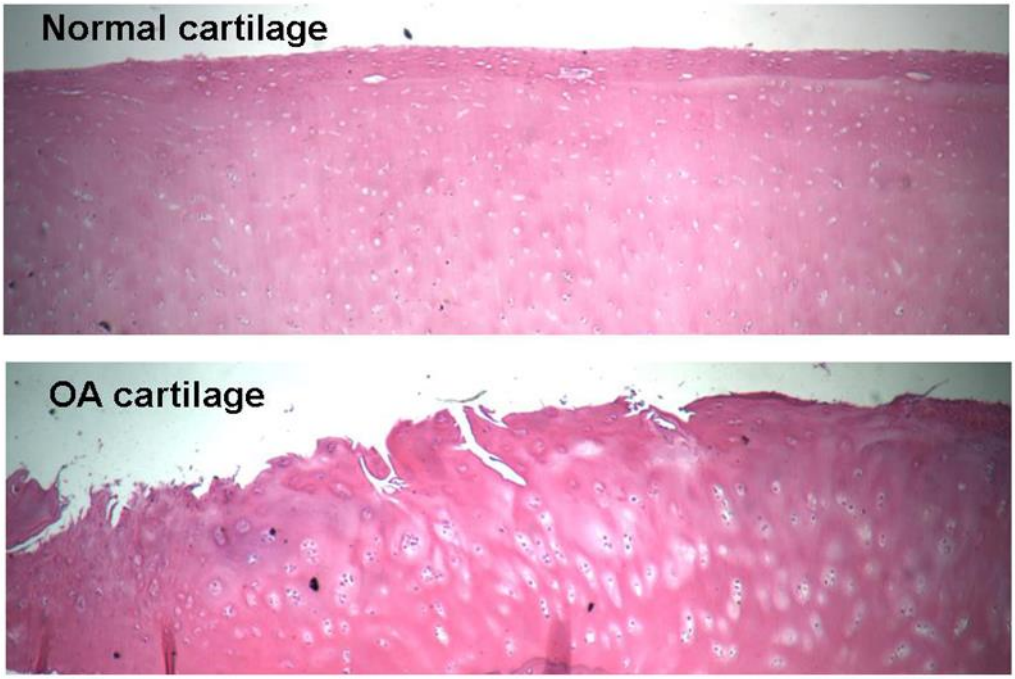



**Figure 1. 3 Histology of normal human and osteoarthritic cartilage.**
Hematoxylin and Eosin Staining. Picture was taken from
*http://medcell.med.yale.edu/histology/connective_tissue_lab/osteoarthritis.php*

Several pro-inflammatory factors have been described as having a role in OA progression. Degradation of collagen or aggrecan results in the formation of so-called “neoepitopes” that can eventually lead to inflammation and progression of the disease (Cibere et al., 2009). Commonly known pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and a variety of chemokines have been reported to have important roles in OA (Kapoor et al., 2011). In addition, recently described Damage Associated Molecular Pattern molecules (DAMPs), which can induce immune responses (Chockalingam et al., 2013; de Seny et al., 2013) have been described. These include Serum Amyloid A (SAA) and Tenascin-C (TN-C). TN-C expression has been described in human chondrocytes (Cheng et al., 2012) although, its role in OA progression has not been well defined.

Other factors, reported to trigger OA include cell apoptosis and free fatty acids (Alvarez-Garcia et al., 2014). In a recent cohort study, which included more than 7,000 OA patients, the authors described several chromosome loci associated with OA. Fat mass and obesity associated gene (FTO) was one of the genes within the described loci. High expression of FTO is correlated with obesity, which could play an important role in OA (Zeggini et al., 2012).

The mechanism of early events leading to the development of OA has not yet been completely described. Several theories have been proposed. Changes in both cartilage and subchondral bone have been suggested as triggers of OA (Bettica et al., 2002; Madry et al., 2012).

Proteins including collagen type II or X, hyaluronan, MMP-13, and others have been proposed to be potent OA biomarkers. Lesions seen in the superficial zone are characterised by fibrillation of cartilage and loss of proteoglycans. There is also a change in chondrocytes phenotype, they become hypertrophic in comparison to healthy, mature, matrix-producing and senescent chondrocytes. The final stage of chondrocyte hypertrophy is calcium deposition. This process, called endochondral ossification, is desirable in the normal growth-plate during bone maturation (Sun et al., 2014). In OA, however, this phenotype change can lead to the matrix reorganisation and inflammation of the cartilage. OA chondrocytes are also reported

to produce more alkaline phosphatase (ALP), which is a typical bone protein (Pfander et al., 2001). Figure 1.4 shows the main symptoms of OA.

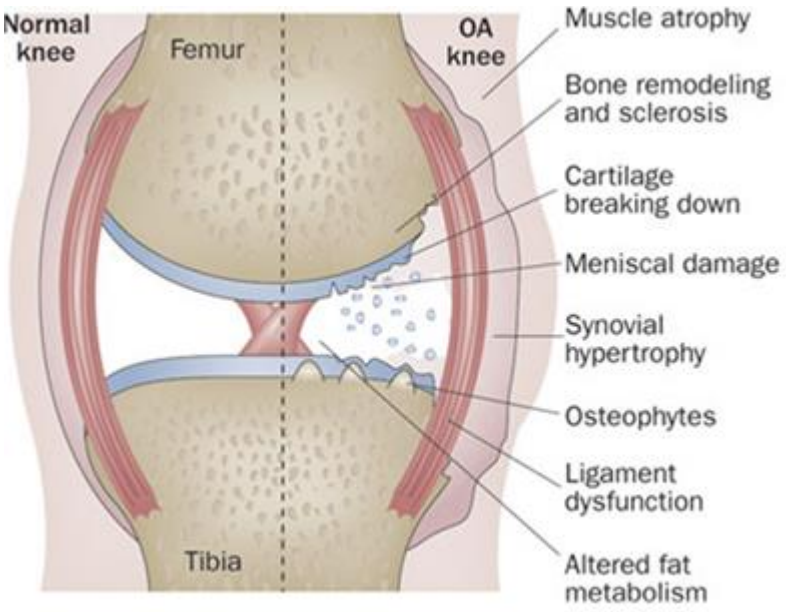



**Figure 1. 4 Knee joint components' contribution to OA pathogenesis.**
Diagram taken from (Hunter, 2011) Pharmacologic therapy for osteoarthritis—the era of disease modification *Nat. Rev. Rheumatol*

## 1.3 Obesity

A number of studies have shown that obesity correlates with the incidence of OA not only in weight-bearing joints including knees (Dougados et al., 1992; Marks, 2007) but also for non-weight bearing joints OA, such as the joints in the hands, as demonstrated by a cohort study (Grotle et al., 2008). Body mass index (BMI) is strongly correlated with knee OA, but only weakly with hip OA (Sturmer et al., 2000).

Fat tissue can no longer be considered just as an energy storage (Kershaw et al., 2004). It is also an effective endocrine organ. Not only does it produce hormones in a paracrine manner, but also is an efficient source of systemically secreted substances. All of the above suggests that adipose tissue could be a potent target in osteoarthritic research.

### 1.3.1 Adipose tissue

#### 1.3.1.1 Types of adipose tissue

Adipose tissue is a connective tissue composed mainly of adipocytes. Two main adipose tissue types exist: White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT). Some recent studies described an intermediate type, so called, Beige Adipose Tissue (Wu et al., 2012). White Adipose Tissue fat cells are highly specialised in the synthesis, storage and release of triglycerides (Lanthier et al., 2014). The diameter of white adipose tissue adipocyte can reach up to 200 μm (Wree et al., 2014). The cell comprises one large or several medium size lipid droplets. In healthy adipose tissue adipocytes are in a sufficient contact with the capillary system.

Brown adipose tissue specialises in energy expenditure and heat generation. It is mainly found in newborns and hibernating animals. Brown adipocytes are very characteristic with several small lipid droplets next to a high number of mitochondria (containing iron), which play an important role in thermoregulation and make it brown (Nam et al., 2015). The brown adipose tissue content decreases with age and is mainly detected in supraclavicular and paraspinal regions in adults (Yoneshiro et al., 2011).

#### 1.3.1.2 Location of White Adipose Tissue (WAT)

White adipose tissue can be divided into several types depending on its location including subcutaneous adipose tissue (SAT) which lies under the skin and omental/visceral adipose tissue (VAT) which lies within the peritoneum. In healthy subjects (with normal weight) the majority of adipose tissue (up to 80%) is subcutaneous and only 10-20% is visceral adipose tissue. SAT can be characterised by a lower number of larger adipocytes while VAT has a higher number of smaller adipocytes. SAT is known to have greater angiogenic properties and higher capillary density than VAT (Lanthier et al., 2014). Whereas VAT has comparatively a higher number of resident macrophages (Amano et al., 2014). Several specific adipose tissue types comprise VAT including pericardial, periaortic, perigonadal and perirenal. All of these fatty tissue depots play a role in organ protection, fatty acid metabolism and healing in the healthy organism but are also very important in the pathogenesis of many diseases for example atherosclerosis (Li et al., 2015).

### 1.3.2 Obesity as a chronic inflammation

Obesity has become a major problem in developed societies. It has been calculated that 50% of British adult will be obese by 2030 (Wang et al., 2011). Obesity is characterised by an excess of WAT due to overfeeding and lack of physical exercise. It is known to increase the likelihood of such diseases as Type 2 Diabetes Mellitus (T2DM), heart conditions (ischemic heart disease, myocardial infarction), stroke (Saydah et al., 2014), OA (Sturmer et al., 2000) and some types of cancer (Bianchini et al., 2002). Obesity is defined based on the body mass index (BMI) or waist-to-hip ratio (WHR). The BMI index is defined as body weight (in kilograms) divided by the square of body height (in meters) and BMI score from 20-25 is considered as normal weight, 25-30 as overweight, 30 to 35 Class I (moderate) Obesity, 35 to 40 Class II (severe) Obesity, above>40 Class III (very severe) Obesity.

Obesity is a low-grade inflammatory status. Fatty tissue is no longer considered to be a passive organ. It produces a huge variety of molecules and contains different populations of cells. In lean subjects, protein production, cell phenotype proliferation, differentiation and cell death are homeostatically maintained. During

weight gain, there is a switch to low-grade inflammation in the whole organism, resulting from the influx of pro-inflammatory macrophages into adipose tissue, together with other cells of the immune system including $CD8^+$ T, mast and B cells (see Figure 1.5) (Kanneganti et al., 2012). Additionally, adipocytes themselves start producing cytokines, and so-called "bad" adipokines. These changes affect other tissues including blood vessels and the pancreas leading to atherosclerosis and T2DM (Osborn et al., 2012).

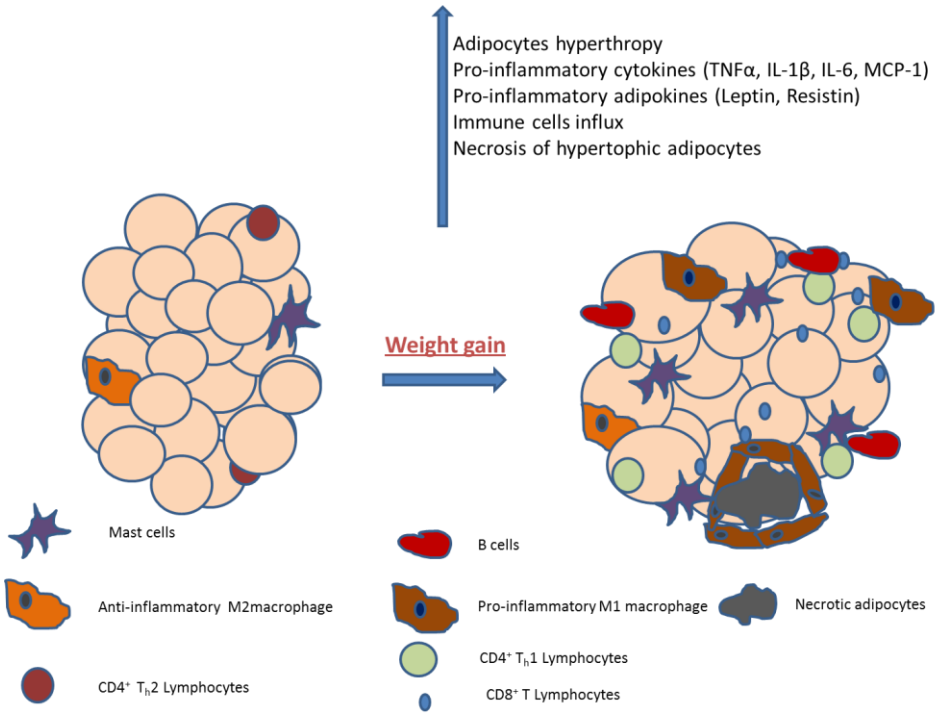



**Figure 1. 5 General scheme of immune changes within the adipose tissue with weight gain.** The difference in proinflammatory markers and adipose tissue leukocytes can be characterized with weight gain. Adapted from (Kanneganti et al., 2012)

Recent studies have demonstrated that obesity has a huge impact on cartilage metabolism (Yaykasli et al., 2015). Increased release of glycosaminoglycans (GAG) has been postulated as a marker of cartilage destruction. A higher content of free GAGs produced by cartilage explants has been positively correlated with BMI of the subject (Buchholz et al., 2010). Another study reported that mRNA for proteins including Peroxisome Proliferator-Activated Receptor α (PPARα), Bone

Morphogenic Protein 7 (BMP-7) and Interleukin 1 β (IL-1β) were highly induced in OA and correlated with BMI (Iliopoulos et al., 2008).

### 1.3.3 Adipokines and cytokines in obesity

Adipokines are proteins, produced by WAT, that have an important autocrine role within fat but also influence other organs in the body. They regulate appetite, energy expenditure and lipid uptake as well as being responsible for glucose and insulin homeostasis. The first adipokine described and most often reviewed is leptin, a product of the *ob* gene in mice (homolog for human *LEP* gene). It is mainly produced by adipocytes and is responsible for the hypothalamic signalling that reduces appetite (McGarry, 1995). The leptin receptor (LEPR) is expressed in the central nervous system (Gautron et al., 2011) and leptin administration increases peripheral glucose intake and reduces appetite (Rossetti et al., 1997). Leptin has many other functions, including regulation of muscle glucose metabolism (Yau et al., 2014), induction of $CD4^+$ T cells proliferation (Cassano et al., 2014), the production of chemokines and macrophage activation (Kiguchi et al., 2009). Mice with genetic knockdown of leptin (or leptin receptor) are obese (*ob/ob* and *db/db* respectively) (Pelleymounter et al., 1995). Although leptin reduces appetite, its expression is increased in human obesity (Considine et al., 1996). Hyperleptinemia in human subjects is explained as the resistance of peripheral tissues to leptin which leads to failure in weight management (Myers et al., 2008).

The second most widely examined adipokine is adiponectin commonly seen as a "good" adipokine. It has anti-inflammatory properties and its level is negatively correlated with obesity. Adiponectin circulates in different multimeric forms and can form trimers, hexamers in addition to high molecular weight multimers (HWM). The HMW form is the most active and important in sensitivity of cells to insulin and in protecting patients from diabetes. Adiponectin is known to inhibit smooth muscle cell proliferation and to decrease the production of some adhesive proteins, which makes it an anti-atherosclerotic factor. It enhances the sensitivity to insulin of many cells and helps maintain the anti-inflammatory macrophage phenotype (Arita et al., 2002; Okauchi et al., 2009; Lovren et al., 2010).

Recent findings suggest that the production of adipokines is not exclusive to adipocytes. Other cell populations (including macrophages) can also be potent producers of adipokines. Pro-inflammatory molecules such as cytokines and chemokines are abundantly produced within fat tissue. Obese patients produce systemically more IL-1β in addition to IL-18 and IL-6 (Syrenicz et al., 2006; Murdolo et al., 2008) as compared to lean individuals. It has been postulated that these proteins have a major influence on obesity-related conditions including atherosclerosis and diabetes (Dalmas et al., 2014; Shi et al., 2015). For instance, IL-1β is a major catabolic factor in cartilage degradation. IL-18 has been reported to induce prostaglandin E2 (PGE2) - an important inflammatory mediator in the synovial membrane while IL-6 is involved in bone remodelling (Iannone et al., 2010).

Although obesity is highly correlated with OA and is considered to be a risk factor for this disease, the role of adipose tissue- derived factors in the progression of OA has not been fully described.

## 1.4 Synovium in OA

Synovial tissue, an important compartment of the knee joint, consists of two layers: intima and subintima. The inner layer – the intima consists of two types of cells: fibroblast-like and macrophage-like synoviocytes. Fibroblast-like synoviocytes (FLS) are important in the secretion of the proteins hyaluronan and lubricin, which maintain the viscosity of the synovial fluid and retain water within the joint. In healthy, normal synovium, the intima does not exceed two layers of cells and is approximately 25 μm thick. The outer layer – the subintima in the knee joint consists of loose fibrous and fatty tissue. The whole structure also includes microvessels and small nerves.

Synovial Fluid (SF) is a viscous fluid found in the synovial cavity. Its main role is to reduce friction between the articular cartilage surfaces found in the joint. In the healthy condition SF consists mainly of hyaluronic acid and lubricin (Hui et al., 2012).

Healthy, intact synovium provides sufficient lubrication for both cartilage-cartilage contact and synovium-cartilage contact. Lubricin and hyaluronic acid reduce friction and provide lubrication. There is an active transport of small plasma proteins (including growth factors) into the joint cavity. Proteins enter the SF via synovial microvasculature but their transport depends on the molecular weight and the charge of the protein (Weinberger et al., 1989). In normal conditions, high-weight molecules including hyaluronic acid and proteoglycans remain within the joint cavity while smaller proteins are able to exit.

In various diseases, this balance is disrupted by reorganisation of the synovial membrane (due to hyperplasia or inflammation), resulting in a noticeable change in the protein concentration in the SF. Proteins produced by the synovium are key players in the pathogenesis of many arthritic diseases including rheumatoid arthritis (RA) (Castro-Santos et al., 2015). Although OA is considered less inflammatory than RA, some authors have reported mild to severe inflammation and synovitis in OA (Haywood et al., 2003).

In synovitis, there are three different pathological changes which have been described within the synovium: 1) hyperplasia 2) stromal activation 3) inflammation (Krenn et al., 2006). Hyperplasia of the synovium is defined as an increased cell number in the intima. Fibroblast-like synoviocytes proliferate and the general intima thickness increases. Activation of the stroma occurs in sublining areas and is defined as slight to severe oedema. The third feature of synovitis is the inflammatory infiltration characterised by slight to severe leukocyte invasion.

Production of pro-inflammatory cytokines including IL-1β, TNF-α, IL-6 and IL-8 by synovium have been reported to be important in OA progression (Sokolove et al., 2013). However, synovium also produces anti-inflammatory cytokines for example IL-4, IL-10 and IL-1Ra (Sutton et al., 2009). Fibroblast-like synoviocytes are very potent producers of pro-inflammatory and pro-catabolic molecules. OA Fibroblast-like synoviocytes produce TNF-α, IL-1β and MMPs (Shen et al., 2014).

Synovium contains not only synoviocytes but also immune cells including macrophages, T-cells, B-cells and mast cells. There is an altered CD4/CD8 ratio of the T-cell subtypes found in OA patients in comparison to healthy people (Revell et al., 1988; Saito et al., 2002). The subtypes of T cells in the synovial membrane are considered to have a unique role in cytokine production and in the pathogenesis of arthritis (Schulze-Koops et al., 2001).

## 1.5 Infrapatellar (Hoffa's) Fat Pad (IPFP) in OA

The infrapatellar fat pad, also known as Hoffa's pad, is located within the knee capsule deep to the patella but outside the synovium. Hoffa's pad occupies dead space within the joint together with two smaller fatty depots called the quadriceps and pre-femoral fat bodies. Those fatty structures maintain the joint cavity and promote efficient lubrication (Morini et al., 1998). The origin of the IPFP is unknown, some authors suggest it is most similar to a subcutaneous fat deposit (Vahlensieck et al., 2002) due to the fibrous structures surrounding the adipocytes. Other authors state that it is more similar to a visceral fat deposit (Ioan-Facsinay et al., 2013).

IPFP is a rich source of mesenchymal stem cells (MSC) with high chondrogenic (Felimban et al., 2014) but lower adipogenic and osteogenic potential in comparison with subcutaneous adipose tissue MSC from the same donor (Lopa et al., 2014). It is also a source of inflammatory cells including leukocytes (Klein-Wieringa et al., 2011) or $CD31^+$ endothelial cells. MSC isolated from OA IPFP had comparable or slightly weaker osteogenic potential in comparison to MSCs from RA patient patients (Skalska et al., 2014).

Some studies have reported a positive role of the IPFP in mediating OA – related pain due to shock absorption (Han et al., 2014), however, the IPFP adjacent to the synovial layer is considered to be an active source of many cells and factors involved in OA (see Figure 1.6). The IPFP produces IL-6 (Ushiyama et al., 2003) at a level almost 2-fold greater than the subcutaneous adipose tissue (SAT) depots. The IPFP also produces 40% less leptin and 70% more adiponectin (Distel et al., 2009)(Gegout et al., 2008). It has been demonstrated as having a potential role in the induction of synovial inflammation in OA patients: IPFP-conditioned medium has been reported to stimulate synovial fibroblast-like synoviocytes to produce IL-6 and IL-8 (both at the mRNA and protein levels) in addition to MMP-1,3,9 and 13 possibly through the action of prostaglandin-2 (PGE-2) (Eymard et al., 2014). The IPFP-conditioned medium was able to stimulate pro-fibrotic properties of these cells by increasing collagen production and by stimulating cell migration (Bastiaansen-Jenniskens et al.,

2013). Conversely, IPFP-conditioned media reduced nitric oxide (NO) production and MMP-1 gene expression in cartilage, inhibited IL-1β mediated up-regulation of MMP-1 and MMP-3 gene expression while stimulating collagen II gene expression (Bastiaansen-Jenniskens et al., 2012).

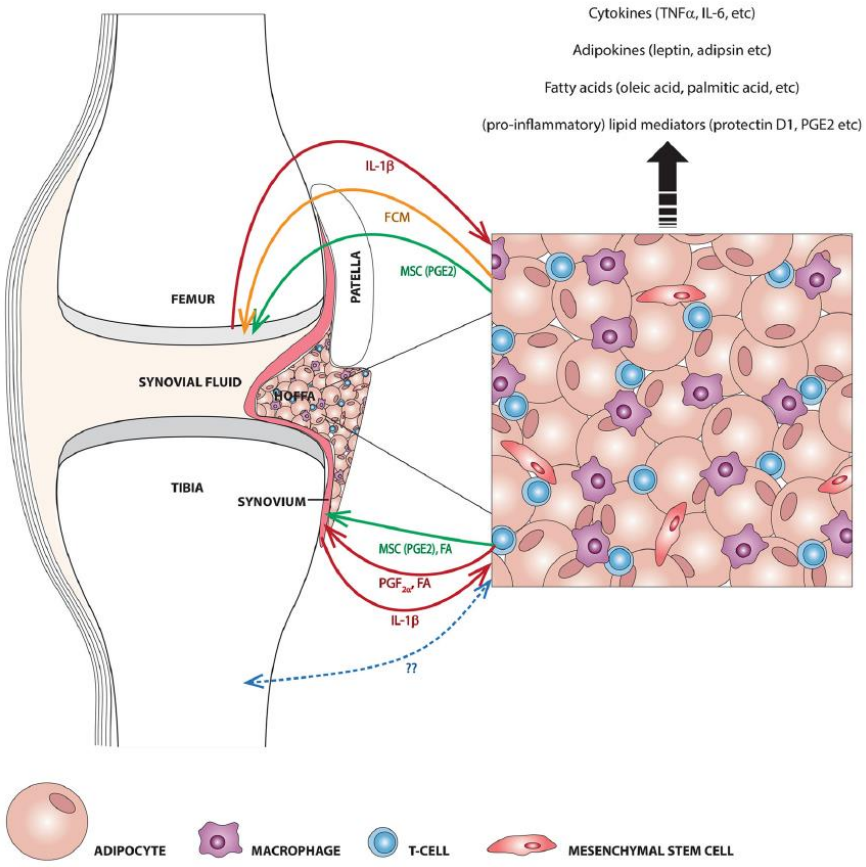



**Figure 1. 6 Infrapatellar Fat Pad Contribution to OA.**
Diagram taken from (Ioan-Facsinay et al., 2013) showing the contribution of IPFP to OA. IPFP is a potent producer of cytokines, adipokines and other obesity relates factors including FFA.

## 1.6 Subchondral bone in OA

### 1.6.1 Bone role, composition, structure and types

Bone is a mineralized connective tissue that supports and protects the whole organism as well as being a very important organ in hematopoiesis. Bone marrow is a crucial hematopoietic milieu and facilities leukocyte maturation. Bone is highly specialized in the deposition of calcium phosphate within the collagen matrix. Calcium phosphate is the main inorganic component of the bone which is organized in a chemical arrangement called hydroxyapatite that provides fracture resistance. The main organic compound of the bone is collagen type I.

There are two main types of bone: cortical and cancellous. Cortical bone (also named compact bone) as its name suggests forms the cortex (outer shell) of bone structure. It is denser, harder and stronger than cancellous bone. The main function of cortical bone is to provide support, protection and to be a specific lever for movement. Cancellous, also called trabecular or spongy bone, is less dense and more porous than cortical bone. It is highly vascular and very often contains red bone marrow where hematopoiesis occurs. It can be typically found at the end of the long bones proximal to joints and in the inside of the vertebrae.

Although bone is the hardest and the most rigid tissue within the body, it is constantly remodelled by two main cell types, osteoblasts and osteoclasts. Osteoblasts specialize in bone matrix formation while osteoclasts are specialized in bone resorption. When osteoblasts become trapped within the bone matrix in lacunae they mature into osteocytes. They are connected to each other via cytoplasmic extensions which lie in canaliculi and exchange nutrients and metabolites through gap junctions. Osteocytes are non-proliferating, fully mature and long-lived cells with an average life span of about 25 years. Osteoclasts are large, multinucleated cells, which originate from macrophages. Their main role is bone resorption and remodelling (Buckwalter et al., 1987). Osteoclasts have the ability to resorb bone and can be found on the bone surface in shallow pits of trabecular bone. They produce specific proteins including tartrate-resistant acid phosphatase (TRAP) or various

lysosomal enzymes and serine proteases. Macrophages Colony stimulating factor (M-CSF) plays a crucial role in osteoclastogenesis (Teitelbaum, 2000). Osteoclast precursor cells express Receptor Activator of Nuclear Factor Kappa-B (RANK) which is activated during binding to osteoblast-specific protein Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL) which leads to osteoclasts activation and bone resorption. RANKL can also bind to its decoy receptor osteoprotegrin (OPG) which is a negative regulator of bone resorption due to blocking RANK and RANKL binding.

### 1.6.2 Subchondral bone

The tissue lying directly under the cartilage is subchondral bone which can be further divided into the subchondral bone plate and trabecular bone. The subchondral bone plate is composed of 1-3 mm corticalised bone. It separates calcified cartilage from the bone marrow-containing trabecular bone. The trabecular compartment of the articular joint is highly vascularized and provides nutrients to the deep zones of cartilage. Subchondral bone by its specific organization is a specialized structure that responds to the loads applied to the joint (Madry et al., 2010).

### 1.6.3 Subchondral bone role in OA

Progression of OA is associated with several changes within subchondral bone. Overloading of the joint is associated with increased microdamage and microfractures of the subchondral bone (Henrotin et al., 2012). The rate of bone remodelling changes during disease progression (Chen et al., 2015). Furthermore, subchondral cortical and cancellous bone are architecturally, physiologically and mechanically different and respond differently to OA (Grynpas et al., 1991). Early stages of OA can be characterized by increased vascularity and reduced bone density while in later stages, there is subchondral sclerosis and decreased bone resorption without decreased bone formation (Klose-Jensen et al., 2015). Furthermore, there is a duplication of tidemark and penetration of the vessels reported during OA pathogenesis in humans (Bonde et al., 2005)

Some animal models, i.e. non-human primates and guinea pigs (Carlson et al., 1994; Huebner et al., 2002) suggest that thickening of the subchondral plate occurs prior to cartilage damage, rather than vice versa.

## 1.7 The role of adipokines in OA

As previously mentioned, adipose tissue is an endocrine organ. It releases cytokines, including IL-1β and tumour necrosis factor-α (TNF-α), and adipokines, including leptin adiponectin, visfatin, resistin, lipocalin and chemerin. The adipokine family affects a wide variety of physiological and pathophysiological processes, including food intake, energy expenditure, lipid and glucose metabolism, inflammation, insulin metabolism and bone formation. Products from adipose tissue are also viewed as potential systemic factors, which link obesity to arthritis. The recent studies concerning the involvement of adipokines in OA will now be discussed.

### 1.7.1 Leptin

Several studies have reported that the serum level of leptin positively correlates with OA (Gandhi et al., 2010; Boer et al., 2012) and have stated that it has an important role in the progression of OA (Vuolteenaho et al., 2014). Leptin is produced in explant cultures of cartilage, infrapatellar fat pad, synovium and osteophytes from the joints of OA patients (Gegout et al., 2008). It can also be found in osteoarthritic chondrocytes (Francin et al., 2011). Normal non-OA cartilage produces considerably less leptin than even mildly OA cartilage (Simopoulou et al., 2007). Boer et al. reported that the plasma level of this protein correlated with other OA biomarkers including C-terminal tellopeptide of type II collagen, monoclonal antibody 846 against chondroitin sulphate (Boer et al., 2012). Hui et al stated that leptin is a pro-inflammatory adipokine, which either alone or with IL-1β, induces collagen release in bovine cartilage by up-regulation of collagenolytic and gelatinolitic activity (Hui et al., 2012). Leptin has also been shown to induce MMP-3, MMP-9, and ADAMTS-4 and -5 production in addition to proteoglycan depletion in chondrocytes but also to induce collagen II mRNA expression in rat cartilage (Bao et al., 2010). Leptin induces production of MMP-1, MMP-3 and MMP-13 in human cartilage (Koskinen

et al., 2011). In addition, higher leptin levels and leptin receptor expression have been reported in areas of more advanced osteoarthritic change within the cartilage of the same joint (both mRNA and protein level) (Simopoulou et al., 2007).

Production of MMP-1 and MMP-13 by chondrocytes is induced by conditioned media from the infrapatellar fat pad (IPFP), what was reduced by blocking leptin with a specific antibody (Hui et al., 2012). Whereas, leptin can also enhance production of pro-inflammatory markers in human chondrocytes, including nitric oxide (NO), prostaglandin E2 (PGE2), IL-6 and IL-8 (Vuolteenaho et al., 2009). Together with adiponectin, leptin can induce Vascular Cell Adhesion Molecule 1 (VCAM-1) expression by chondrocytes (Conde et al., 2012).

The level of leptin in synovial fluid (SF) is increased in obese OA patients while its circulating inhibitors (sOb-b and SOC-3) are decreased (Vuolteenaho et al., 2012). In chondrocytes from obese patients, unlike those from lean patients, high doses of leptin induce Insulin-like Growth Factor 1 (IGF-1) and collagen II (Pallu et al., 2010). On the basis of these studies, a potential role of leptin in the local progression of OA has been postulated and it has been suggested that decreasing the level of leptin by weight reduction could be a mechanism for improved physical function and reduced symptoms in OA patients (Richette et al., 2011).

### 1.7.2 Leptin receptor

The leptin receptor (Ob-R) is encoded by the *db* gene in rodents (LEPR in humans) and belongs to the class I cytokine receptor superfamily (Zabeau et al., 2004). It has 6 isoforms, the longest of which is Ob-Rb, expressed by several different cell types including pancreatic cells, epithelial cells and T lymphocytes (Park et al., 2001; Papathanassoglou et al., 2006; Morioka et al., 2007). The soluble form of Ob-R is present in joint fluids (Vuolteenaho et al., 2012). Vuolteenaho et al have demonstrated that leptin and Ob-R level is significantly induced in both synovial fluid and serum in severe OA. Although many studies have confirmed the pro-inflammatory role of leptin in OA, Griffin et. al. showed that, even though a high-fat diet-induced OA in C57B/6 mice, direct injection of leptin into the joint did not induce alteration in synthesis of extracellular matrix by healthy cartilage,

indicating that leptin was not a direct mediator of OA (Griffin et al., 2010). Of particular interest was the finding that obese, leptin-deficient mice (*ob/ob*) or leptin-receptor-deficient mice (*db/db*) do not develop spontaneous OA (Griffin et al., 2010).

### 1.7.3 Adiponectin

Adiponectin is 30kDa protein that possesses several Gly-X-Pro repeats next to a cluster of Gly-X-Y domains which altogether forms collagen-like domains. Furthermore, the C-terminal aminoacids sequence form globular domain (Scherer et al., 1995; Nakano et al., 1996). Adiponectin is known to circulate in different molecular oligoforms (monomer, trimer and multimers) (Kadowaki et al., 2005) and a shorter, cleaved globular form. Adiponectin needs to be glycosylated in order to gain biological activity (Chandran et al., 2003). The monomeric form of adiponectin is not detected in the circulation and the globular form can only be found in small quantities (Wedellova et al., 2013). Fully functional adiponectin is a trimer. Four to six trimers associate to form a high-molecular-weight structure (HMW adiponectin), which is the predominant form of adiponectin in blood. It is a highly expressed protein within adipose tissue, which circulates in the bloodstream in high concentrations. It constitutes approximately 0.01% of the total of plasma proteins and its serum level correlates negatively with obesity (Arita et al., 1999).

Obesity is associated with both a decrease of adiponectin and adiponectin receptor (ADIPOR1 and ADIPOR2) levels within adipose tissue and serum. Down-regulation of ADIPOR1 and ADIPOR2 in the liver is associated with the development of T2DM (Yamauchi et al., 2001; Yamauchi et al., 2007). Boer et al showed that the adiponectin level in serum of OA patients negatively correlated with BMI, but surprisingly, its synovial fluid level correlated with pro-inflammatory cytokines including IL-1β (Boer et al., 2012). Adiponectin is also produced by cartilage, the infrapatellar fat pad, synovium and osteophytes in OA patients (Gegout et al., 2008). Both adiponectin receptors can be found to be expressed by chondrocytes (Chen et al., 2006). ADIPOR2 is expressed in all cartilage layers while ADIPOR1 within the superficial layer. An increased expression of ADIPOR1 and ADIPOR2 has been reported in OA cartilage lesions (Kang et al., 2010).

Serum level of adiponectin exceeded its level in the synovial fluid (Presle et al., 2006). Both serum and synovial fluid adiponectin level conversely correlate with OA cartilage damage (Honsawek et al., 2010). The ratio of leptin and adiponectin level in serum has been found to correlate with pain in OA patients (Bas et al., 2014). Circulating plasma level of adiponectin have been reported to correlate with the OA marker: cartilage oligomeric matrix protein (COMP). Further, there were variances in adiponectin secretion among patients with different levels of severity of OA (King et al., 2014). Production of adiponectin by OA cartilage positively correlates with PGE2 and MMP-13 (Francin et al., 2014). Adiponectin induces secretion of IL-6, MMP-3, MMP-9 and MCP-1 in chondrocyte cell lines in a dose-dependent manner and induces an important proinflammatory molecule: inducible Nitric Oxide Synthase 2 (iNOS2) production (Lago et al., 2008). The role of adiponectin in OA is controversial. Some authors suggest it is protective (Chen et al., 2006) thus anabolic while others (Kang et al., 2010) report that it is a pro-inflammatory factor. Some of the variation might be accounted by the differences in circulating level of different oligomers, which each play distinct roles. Once ADIPOR1 or ADIPOR2 have been stimulated distinct signalling cascades mediate their signal. However, currently, the role of adiponectin in morbidly obese patients with OA has not been elucidated.

### 1.7.4 Visfatin, Resistin, Lipocalin 2 and Chemerin

Visfatin was first discovered as a cytokine promoting B-cell maturation hence its other name: pre-B-cell colony-enhancing factor 1 (PBEF1) (Samal et al., 1994). Later studies revealed that the same gene encodes an important intracellular enzyme called nicotinamide phosphoribosyltransferase (NAmPRTase or NAMPT) (Wang et al., 2006). It catalyses the transfer of a phosphate group onto nicotinamide D-ribonucleotide and plays an important role in NAD and NADH synthesis pathways. Its third name – visfatin came from the study describing its exclusive expression in visceral fat (Fukuhara et al., 2005). This was contradicted as other studies showed it is also produced in other tissues including subcutaneous adipose tissue (Sitticharoon et al., 2014) and liver (Dahl et al., 2010).

Elevated serum and synovial fluid levels of visfatin have been described in OA patients (Chen et al., 2010). This adipokine is highly expressed by osteophytes (Gegout et al., 2008) and can be found in osteoarticular chondrocytes. Visfatin has been also reported to inhibit IGF-1-mediated proteoglycan synthesis and to inhibit production of collagen II (Yammani et al., 2012). Administration of visfatin enhances prostaglandin 2 (PGE2) production, MMP-3, MMP-13 and ADAMTS-4 and -5 expression in murine chondrocytes (Gosset et al., 2008). The production of these latter proteins correlates with degradation markers of collagen and aggrecan including CTX II in cartilage (Duan et al., 2012).

Resistin is a pro-inflammatory mediator produced by adipose tissue, macrophages and neutrophils. Serum resistin levels positively correlate with obesity and inflammatory state (IL-1β, TNF-α production) in OA patients, but does not correlate with cartilage loss (Boer et al., 2012). There is also a correlation between serum level of resistin and radiographic changes in hand OA (Choe et al., 2012). Resistin levels are higher in serum than in synovial fluid (Presle et al., 2006) and this protein is produced by cartilage, infrapatellar fat pad, synovium and, in small amounts, by osteophytes within the joint of OA patients (Gegout et al., 2008).

Lipocalin 2 (LCN2), also termed siderocalin or neutrophil gelatinase-associated lipocalin (NGAL), is mainly produced by adipose tissue but can also be expressed in neutrophil granules and can be found in chondrocytes . It forms a complex with MMP-9 and is believed to be involved in the pathogenesis of OA (Owen et al., 2008). Its expression can be regulated by leptin, adiponectin and IL-1β (Gupta et al., 2007) in addition to dexamethasone (Owen et al., 2008) . Higher LCN2 expression was reported in hypertrophic chondrocytes (Gupta et al., 2007). Toll-like receptor4 (TLR4) activation can stimulate LCN2 expression in chondrocytes (Gómez et al., 2013).

Chemerin was first described in psoriasis patients treated with tazarotene (retinoid approved for treatment of psoriasis and acne). This protein is secreted by many cell types including adipocytes and epithelial cells. It induces chemotaxis of cells expressing the chemerin receptor Chemokine-Like Receptor-1 (CMKLR1) (Kaur et

al., 2010). It recruits dendritic cells and macrophages to sites of inflammation (Skrzeczynska-Moncznik et al., 2009). Chemerin expression is highly induced during adipocyte differentiation and has an influence on lipolysis (Goralski et al., 2007). Chemerin is also present in the aorta wall in atherosclerosis and is considered as a highly pro-inflammatory protein. High levels of chemerin have been described in obese patients (Shin et al., 2012). The role of chemerin in OA is not fully described. Although it is known that synovial cells express both chemerin and CMKLR1 and chemerin is present in synovial fluid, it does not have chemotactic properties for synoviocytes. Moreover, it stimulates TLR4 gene expression and CCL2 synthesis in synoviocytes (Eisinger et al., 2012). Chemerin levels are higher in the serum than in the synovial fluid of OA patients and its level in the synovial fluid of knee OA patients is correlated with disease severity evaluated with the Kellgren-Lawrence (KL) grading (Huang et al., 2012).

In summary, all aforementioned adipokines have been suggested in the literature to be potent players in OA. However, there are still many questions about the mechanisms and pathways they may be involved in. An understanding of how the levels of all of the adipokines and their receptors correlate with obesity in OA patients would be informative.

### 1.7.5 Subchondral bone and adipokines

The role of obesity in OA was firstly ascribed to overloading of the joints. However, studies now suggest that obesity-associated OA is not purely due to the excessive loading (Collins et al., 2015). Obesity has been reported to have a beneficial effect on bone (Ribot et al., 1987; Felson et al., 1993) but more recent studies indicate that excess weight can have a negative effect on bone biology (Sukumar et al., 2011; Ootsuka et al., 2015). Obesity is known to create a chronic inflammatory state with a higher level of circulating pro-inflammatory cytokines, which may promote osteoclast activation (Cao et al., 2010). Cytokines including IL-1β or TNF-α are elevated in obesity and are also potent activators of osteoclasts (Hofbauer et al., 1999).

Adipokines, are now considered to be important factors in the bone formation and remodelling (Zhang et al., 2013). Although, first described to be mainly produced by adipose tissue, adipokines including (1) leptin (Gordeladze et al., 2002), (2) resistin (Thommesen et al., 2006) and (3) adiponectin (Berner et al., 2004; Huang et al., 2004) have now been reported to be expressed by both osteoblasts and osteoclasts.

Leptin has been postulated to have a dual role in bone biology: mice lacking leptin (*ob/ob*) are obese and exhibit high bone density and leptin is able to stimulate loss of trabecular and cortical bone mass when administered locally (Steppan et al., 2000). Other studies have reported that leptin upregulates osteoblastogenesis, osteoblast proliferation and matrix synthesis (Gordeladze et al., 2002; Holloway et al., 2002) while inhibiting osteoclastogenesis in vitro. Another study has shown that genetically obese mice lacking leptin display a decreased trabecular volume in long bones, but increased vertebral bone mass which was reduced by leptin administration (Heep et al., 2009).

Mutabaruka et al have shown that subchondral bone from OA patients produces more leptin than bone from normal subjects (Mutabaruka et al., 2010). In addition, leptin is able to stimulate its own production in subchondral bone and the proliferation of osteoblasts. Inhibition of leptin synthesis is associated with a reduced level of ALP. It has been suggested that the leptin and leptin receptor (Ob-Rb) signalling pathway is responsible for the elevated ALP activity in OA. There are opposing results on the correlation of circulating levels of leptin with human bone mass: serum leptin levels correlate with bone mass in obese women but do not correlate with markers of bone resorption/formation (Goulding et al., 1998). In contrast, some studies report weak or no correlation (Iwamoto et al., 2000; Yilmazi et al., 2005), whilst others, even report a negative correlation with BMI (Sato et al., 2001). These differences may be due to study designs in these reports e.g. women vs men, obese vs. osteoporotic, pre- vs. post-menopausal subjects.

The adiponectin serum level is an important factor in bone biology and is associated with an increase in trabecular bone mass (Oshima et al., 2005) due to a reduction in bone resorption activity of osteoclasts and possibly also due to inducing osteoblast

activity. Studies in mice showed that knockout of adiponectin has an impaired osteoblastogenesis and mineralization (Aguirre et al., 2014). Mice with up-regulated AdipoR1 expression exhibit increased bone volume and number of trabeculae than wild-type mice (Lin et al., 2014). Adiponectin increased trabecular bone mass and inhibited RANKL-mediated osteoclastogenesis of macrophages obtained from peripheral mononuclear blood cells (PMBC) in mice (Oshima et al., 2005).

Visfatin level has been reported to be negatively correlated with BMD (Sucunza et al., 2009) in acromegaly. Visfatin is produced in a high amount by osteophytes (Chen et al., 2010) and is also a potent stimulator of IL-6 and IL-8 production by osteoblasts (Laiguillon et al., 2014).

Resistin is expressed by osteoblasts and osteoclasts (Thommesen et al., 2006) and stimulates IL-6 expression and weakly stimulates OPG or RANKL. Lipocalin 2 is shown to be up-regulated during osteoclastogenesis but down-regulated in osteoblastogenesis (Rucci et al., 2015). Neutralisation of chemerin blocks osteoclastogenesis (Muruganandan et al., 2013) and augments osteoblastogenesis while reducing adipogenesis (Zabel et al., 2006; Muruganandan et al., 2010).

The main findings of the role of adipokines in the pathogenesis of OA are summarised in Figure 1.7.

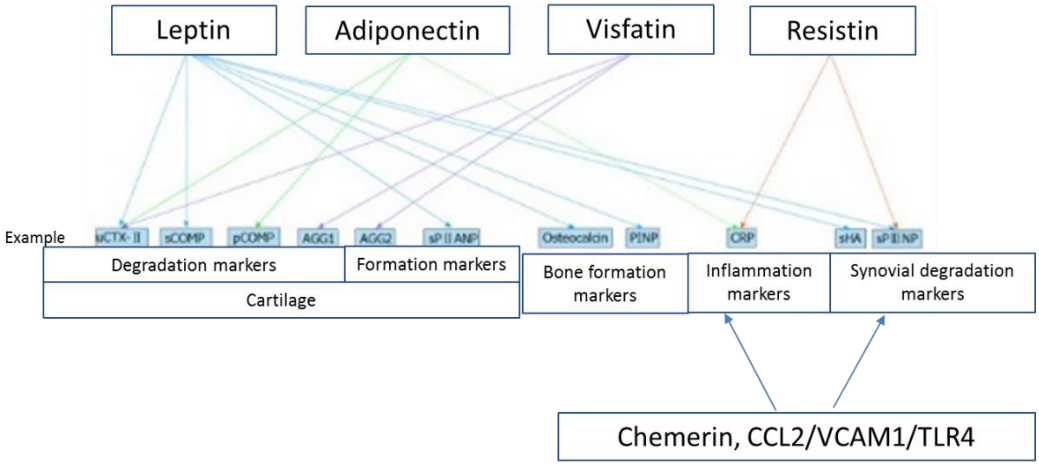



**Figure 1. 7 The main reported findings of the role of adipokines and adipose-tissue related factors in the pathogenesis of OA.**
Diagram adapted from (Poonpet et al., 2014).

## 1.8 Hypothesis and Aims

Although OA and obesity are widely studied diseases, how they interrelate is not properly described and interpreted. Adipokines and adipose-derived cytokines have been reported to play a role in the pathogenesis of OA, however, the differences between obese and lean individuals with OA has not been well studied. Obesity has become a major problem in developed societies. It has been calculated that 50% of British adults will be obese by 2030 (Wang et al., 2011). Fat tissue can no longer be considered just as an energy store (Kershaw et al., 2004). It is also an effective endocrine organ. All of the presented data suggests that adipose tissue could be a potent aim in osteoarthritic research. A systemic role for adipose tissue-secreted proteins (adipokines) has been widely reported. Nonetheless, their local production within the knee joint hasn't been well characterised. All of the above lead to the main hypothesis in this study: that the lean and obese patients with OA differ on the molecular, cellular and structural level in the local tissues within the knee joint. The main aims to prove that were:

- To investigate the production of obesity-related markers (on gene and protein level) by tissues within the knee joint mainly:
  -Cartilage and chondrocytes
  -Synovium
  -IPFP
  -Osteoblasts
- To characterise the immune cell profile of local adipose tissues surrounding the knee joint with regards to obesity.
- To investigate local changes in bone microstructure in lean and obese OA patients.

# Chapter 2: Materials and methods

## 2.1 Materials:

### 2.1.1 List of reagents

**Table 1 List of reagents**

Tissue and cell isolation/cell culture reagents

| Product | Company |
|---|---|
| PBS (Phosphate buffered saline) | SigmaAldrich |
| 0.01% Tween 20 ® (Polysorbate 20 Surfactant) | SigmaAldrich |
| Trypsin with 0.25% EDTA (EthyleneDiamineTetraacetic Acid) | Life Technologies |
| Collagenase II | Life Technologies |
| IMDM (Iscove's Modified Dulbecco's Medium) | Life Technologies |
| DMEM (Dulbecco's Modified Eagle Medium) | Life Technologies |
| Trypan Blue | Fluka |
| 100x PenStrep (Penicilin/Streptomycin) | Life Technologies |
| 100x Anti/Anti (Fungizone+ Penicilin/Streptomycin) | Life Technologies |
| FBS (Fetal Bovine Serum) | Life Technologies |
| Collagenase I | Life Technologies |
| HBSS (Hanks' Balanced Salt Solution) | SigmaAldrich |
| Red Cells Lysis Buffer | BD Bioscience |

RNA isolation/DNA synthesis/agarose gel electrophoresis/PCR reactions reagents

| | |
|---|---|
| Qiazol | Qiagen |
| Chloroform | SigmaAldrich |
| Isopropyl alcohol | SigmaAldrich |
| Ethanol | SigmaAldrich |
| DNA/RNA-se free water | SigmaAldrich |
| Qiagen Mini RNA isolation kit | Qiagen |
| RNA Denaturating Loading Buffer | New England Biolabs |
| Agarose | SigmaAldrich |
| MOPS (3-(N-morpholino)propanesulfonic acid) | SigmaAldrich |
| EDTA (Ethylenediaminetetraacetic acid) | SigmaAldrich |
| Sodium acetate | SigmaAldrich |
| Formaldehyde | SigmaAldrich |
| iScript | BioRad |
| DNAse I kit | Life Technologies |
| TopTaq | Qiagen |
| SYBRStain | Life Technologies |
| Tris Base | SigmaAldrich |
| DNA ladder | BioLabs |
| iTaq Master Mix | Bio-Rad |

Protein isolation/SDS-PAGE/Western Blot reagents

| | |
|---|---|
| Protease inhibitor | Thermo Scientific |
| Phosphatase inhibitor | SigmaAldrich |
| 10xRIPA buffer (RadioImmunoPrecipitation Assay) | Millipore |
| BSA (Bovine Serum Albumin) | SigmaAldrich |
| Bradford Reagent | Bio-Rad |
| Acrylamide/Bis-Acrylamide 19:1 (40%) | SigmaAldrich |
| Ammonium Persulfate | SigmaAldrich |
| TEMED *(N,N,N′,N′-* TEtraMEthylethyleneDiamine) | SigmaAldrich |
| 2x Laemmli Denaturating Buffer | SigmaAldrich |
| Protein marker (PageRuler) | ThermoScientific |
| Methanol | SigmaAldrich |
| Odyssey Blocking Buffer | Li-COR |
| Boric Acid | SigmaAldrich |
| SDS (Sodium Dodecyl Sulfate) | SigmaAldrich |

ELISA reagents

| | |
|---|---|
| Human Total Adiponectin/ARP Quantikine ELISA kit | R&D System |
| Human MCP-1 Mini Elisa Development Kit | Peprotech |
| ABTS Liquid Substrate ( 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) | SigmaAldrich |

IHC/IF/ Flow Cytometry reagents

| | |
|---|---|
| Citric Acid Monohydrate | SigmaAldrich |
| Sodium citrate | SigmaAldrich |
| 30% Hydrogen Peroxidase solution ($H_2O_2$) | SigmaAldrich |
| Dako Blocking Solution | Dako |
| Dako Diluent | Dako |
| DAB(3,3'-Diaminobenzidine) Buffer | Cell Signalling |
| Harris Hematoxylin solution | SigmaAldrich |
| $MgSO_4$ | SigmaAldrich |
| $NaHCO_3$ | SigmaAldrich |
| HistoChoice | SigmaAldrich |
| DPX mounting medium | Fluka |
| Formaldehyde | SigmaAldrich |

## 2.1.2 List of solutions

**Table 2 List and recipe of solutions.**

| Buffer/Solution | Recipe |
|---|---|
| 10xTBE buffer | 108g Tris Base, 55g Boric Acid, 40ml 0.5M EDTA, dd$H_2O$ up to 2 litres adjusted for pH=8.0 |
| 10xMOPS | 41.9g MOPS, 8.2g sodium acetate, 3.72g EDTA up to 1l dd$H_2O$ adjusted for pH=7.0 with NaOH |

| | |
|---|---|
| 1xMOPS Running buffer | 100ml 10xMOPS buffer, 20ml 37% formaldehyde, 880ml dd$H_20$, stored in the dark. |
| Protein lysis buffer | 880µl PBS, 100µl 1xRIPA buffer, 10µl 100x protease inhibitor cocktail, 10µl 100x phosphatase inhibitor cocktail for 1ml |
| Separating buffer | 90.75g Tris, 2.5g SDS and 500ml dd$H_2O$ up to 500ml for pH=8.8 |
| Stacking Buffer | 30.25g Tris, 2.5g SDS and 500ml dd$H_2O$ up to 500ml for pH=6.8 |
| 10x Electrode buffer: | 30g Tris, 72g Glycine, 10g SDS stored at 4$^oC$ for 1l |
| Transfer Buffer | 3.03g Tris, 14.4g Glycine, 200ml Methanol and 800ml dd$H_2O$ for 1l |
| Peprotech ELISA Blocking Buffer | 2g BSA in 100ml PBST. (2%BSA in PBST) |
| Peprotech Diluent | 0.1g BSA in 100ml PBST (0.1% BSA in PBST). |
| 0.1M Citric acid | 21g of citric acid monohydrate up to 1l of dd$H_2O$ |
| 0.1M Sodium Citrate | 29.4g of sodium citrate up to 1l of dd$H_2O$ |
| Citric Buffer | 18ml of 0.1M of Citric acid and 82 ml of 0.1 M sodium citrate diluted in 1l of distilled water at pH=6 |
| Scott's Tap Water | 30g of $MgSO_4$, 2g of $NaHCO_3$ up to 3l of dd$H_2O$ |

# 2.2 Methods:

## 2.2.1 Patient recruitment.

Samples from consented patients undergoing primary total knee replacement surgery (TKR) for OA were collected. Local approval was granted for the study by the Lothian Research Ethics Committee and NHS Lothian Research and Development Management. Patients' summary is shown in Table 4:

**Table 3 Patients summary**

| Technique | Nr of analysed samples | BMI | Age [years] | Female/Male |
|---|---|---|---|---|
| RT PCR | 26 | 32±8 | 68±7 | 19/7 |
| qRT PCR | 20 | 32±10 | 65±11 | 15/5 |
| WB | 20 | 32±8 | 68±8 | 13/7 |
| IHC | 13 | 32±11 | 66±9 | 9/4 |
| Flow Cytometry | 36 | 32±9 | 67±10 | 18/18 |
| µCT | 24 | 35±10 | 67±6 | 13/11 |

## 2.2.2 Tissue preparation and storage

Tissues obtained during TKR were harvested by the surgeon and stored in Dulbecco Modified Media with an antimicrobial and antifungal solution (1xAnti/Anti) solution at 4$^{o}$C for up to 1 hour after the surgery. All tissue explants for RNA and protein analysis were snap-frozen in Liquid Nitrogen and stored at -80$^{0}$C until testing.

## 2.2.3 Cell culture

All cell culture work was performed in laminar flow hoods and cells were cultured at 37$^{0}$C with 5% $CO_2$ in a specific medium.

#### 2.2.3.1 Media and cell culture solution

Anti-Anti: Penicillin 100 U/ml Streptomycin 100µg/ml and Fungizone 25ng/ml

Chondrocyte primary medium: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and Anti-Anti solution.

Standard chondrocytes medium: IMDM supplemented with 10%FBS and 100 U/ml/100µg/ml Penicillin/Streptomycin (P/S).

Osteoblast media: Dulbecco's Modified Essential Medium (DMEM) supplemented with L-Glutamax 10%FBS and 100 U/ml/100µg/ml P/S.

Serum Free media: IMDM with 10%FBS and 100 U/ml/100µg/ml P/S, without FBS.

#### 2.2.3.2 Isolation of chondrocytes from human femoral condyles and human tibial plateaus

Cartilage from end-stage OA patients (OA diagnosed by an orthopaedic surgeon based on X-ray) were collected using sharp dissection to remove it from the tibial plateaus and the femoral condyles of the knee joints (n=20 or different when stated). Residual macroscopically non OA-affected-looking cartilage was selected, pooled and cut into small square pieces approximately 1-2mm$^2$ and soaked for up to 1.5 hour in antimicrobial and antifungal solution (Anti-Anti) at room temperature. Cartilage fragments were then washed twice with sterile PBS and incubated for 30 min in 10 ml of 0.25% Trypsin with EDTA at 37$^0$C with 5% $CO_2$. Trypsin was removed and the cartilage pieces were washed twice with sterile PBS and incubated overnight in 10ml 4mg/ml Collagenase II in IMDM media in 37$^0$C with 5% $CO_2$. After complete digestion of the cartilage, the cells were collected in 50ml falcon tubes and strained through a sterile metal strainer, washed with PBS, centrifuged 5min at 300g. The remaining pellet was rinsed with PBS twice and centrifuged, after straining through a 70µm strainer (Fisher Scientific, UK) cells were resuspended in 1ml IMDM, counted using Trypan Blue and seeded dependent on the size of the culture vessel. For 75cm$^2$ flask 3x10$^6$ cells were plated. For 6-well plate 380.000 cells per well were plated.

Cells were cultured for two days in chondrocyte primary medium (supplemented additionally with Fungizone) which was replaced on day 3 with standard chondrocyte medium.

#### 2.2.3.3 Isolation of osteoblasts from human femoral condyles

The osteoblast were obtained from minimally OA-affected subchondral bone from lateral patellofemoral compartment of the knee. Bone was cut into approximately 2-3mm sized pieces using rongeurs and forceps to remove any bone marrow. Bone chips were shaken vigorously in PBS, allowed to settle and the PBS decanted off and replaced with fresh PBS. The procedure was repeated 4 to 5 times until all visible bone marrow had been cleared. The bone fragments were then placed in 75cm$^2$ culture flasks containing 20ml osteoblast media. Bone chips were incubated for 7 days without disturbance. The medium was then changed and the pieces were incubated for another 7 days, after 14 days the media was changed every 2-3 days. Once the cells reached confluence after 3-4 weeks of culture 3 ml of Trypsin-EDTA solution was added to harvest the adherent osteoblasts (for up to 5 min at 37$^0$C), 10ml of osteoblast media was added and the suspension was collected and centrifuged at 250g for 5 min at room temperature. The supernatant was discarded, the cell pellet re-suspended in 1ml of osteoblast media before being passed through 70µm filter. The cells were counted with a haemocytometer and Trypan Blue and seeded at the required density, usually at 5000 cells/cm$^2$. The cells were maintained up to the 5$^{th}$ passage.

### 2.2.4 Purification of total RNA from human primary cells (chondrocytes, osteoblasts) and tissues (synovium, IPFP)

#### 2.2.4.1 Purification of total RNA from primary cells (chondrocytes and osteoblasts)

Phenol/chloroform based techniques and commercially available kits were used to isolate RNA from cells and tissues. The standard Qiazol method was used for mRNA isolation from chondrocytes and osteoblasts. Confluent cells were washed once with sterile PBS. 1 ml of Qiazol per 75 cm$^2$ flask (or 500 µl per well for 6-well plates)

was added to the flask and scraped with a cell scraper to ensure the removal of any cell material adherent to the flask. The suspension was then incubated at RT for 2 minutes and spun down at 10,000g for 3 minutes to remove insoluble debris. 200µl of Chloroform per 1ml QiaZOL was added to the supernatant after debris disposal. The suspension was mixed by vortexing for 30 seconds, incubated for 2 minutes at RT and centrifuged at 10,000g for 20 minutes. Following the centrifugation, the mixture separated into three phases: a lower red phenol-chloroform phase containing proteins, an inter-phase containing DNA and a colourless upper aqueous phase containing RNA. For mRNA extraction the aqueous phase was collected and gently removed to another sterile 1.5ml RNAse-free tube. An equal volume of isopropyl alcohol was added, mixed well, incubated for 10 minutes at RT and centrifuged at maximum speed for 10 minutes. The supernatant was then discarded and the remaining pellet was washed twice with 1 ml of 70% ethanol and centrifuged for 5 minutes at max speed. After the centrifugation, the 70% ethanol was discarded and the pellet was air-dried for 5-10 minutes and dissolved in RNAse/DNAse free water.

#### 2.2.4.2 Fatty tissues (synovium and IPFP) mRNA isolation by combination Qiazol and Qiagen Mini RNA isolation kit

With fatty tissues, the mRNA Qiazol technique appeared to be insufficient for obtaining clean, non-degraded mRNA. However, the combined Qiazol and Qiagen Mini RNA isolation kit technique yielded good RNA purity and stability. 1ml of Qiazol was added to up to 200mg of synovium or IPFP (snap frozen in Liquid Nitrogen (LN2) and homogenised by mortar and pestle). The suspension was then incubated at RT for 2 minutes, vortexed vigorously several times and spun down at 10,000g for 3 minutes to remove insoluble debris. 200µl Chloroform was added to the supernatant after debris was removed. The suspension was mixed by vortexing for 30 seconds, incubated for 2 minutes at RT and centrifuged at 10,000g for 20 minutes. Following centrifugation, the upper aqueous phase was transferred to a new Eppendorf and an equal volume of 100% ethanol was added. All of the mixture was then transferred to the column provided by the supplier (Qiagen Mini RNA isolation kit) and spun at >10.000g for 15 seconds. The column was then washed by adding 700 µl of the 'RWI©' washing buffer, spun at 10.000 g for 15 seconds,

followed by addition of 500 µl RLT buffer and centrifugation for 15s and then for 2 min at 10.000g. Finally, mRNA was recovered from the column by adding 20-50 µl of RNAse-free water and spinning >10.000 g for 1 min.

#### 2.2.4.3 RNA yield and purity by NanoDrop spectrophotometer

RNA yield and purity were assessed by spectrophotometry using a NanoDrop spectrophotometer (Labtech, UK). The absorbance was measured at wavelength 260, 280 and 230 nm. The absorbance ratio of RNA samples at 260/280nm in a range of 1.7-2.1 and absorbance ratio at 260/230nm 1.7-2.1 were considered as highly pure.

#### 2.2.4.4 RNA stability check

The integrity of RNA was checked by running a RNA Denaturating agarose gel 5 µg of RNA was dissolved in 2xRNA Denaturating Loading Buffer and incubated 5min at $65^0$C. A Denaturating Agarose Gel was prepared by adding 0.5g agarose to 36ml of dd$H_2O$ and microwaving for 2-3 min in order to dissolve the agarose. 14ml of MOPS Running Buffer was added to the pre-cooled agarose under the fume hood, and 1µl of 10,000x concentrated SyberSafe stain (Invitrogen) which was used to visualize the RNA under the ultra-violet (UV) image analyser. The gel was allowed to cool down. Electrophoresis was conducted in 1xMOPS Buffer in horizontal gel electrophoresis system (Bio-Rad). An example of RNA stability test see Appendix 1. Two main bands of RNA were detected 28S rRNA and 18S rRNA (Appendix 1). RNA was stored in $-80^0$C.

### 2.2.5 cDNA synthesis and DNAse I treatment

A total of 1µg of RNA was reverse transcribed. To remove genomic DNA contamination the RNA was treated with 1 µl of DNAse I and 1µl of 10x DNAse I buffer for 15 minutes at room temperature. To stop the reaction, 1µl of 200µM EDTA was added and samples were further incubated at $65^0$C for 10 min in order to deactivate DNAse I activity. cDNA synthesis was prepared according to the iScript manufacturers protocol: 1µl of RT enzyme, 4 µl of 5x RT Buffer (which contained random oligo-dT) and 2 µl of water was added to the sample and incubated

in a thermocycler in following temperatures: 5min in $25^0$C, 30 min at $42^0$C and 5 min in $85^0$C. Samples of cDNA were stored in $-20^0$C.

## 2.2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was prepared according to the protocol provided by the manufacturer. The TopTaq polymerase reaction was prepared by adding 12.5 µl of TopTaq Buffer (2x), 2.5 µl TopTaq Dye (10x), 1 µl diluted cDNA (10-25ng), 2 µl of mixed primers of interest (Forward and Reverse both 10µM), 7µl RNAse free water. The PCR reaction was conducted as follows:

| | | | |
|---|---|---|---|
| Initial Denaturation Temperature: | 95 °C - | 3 min | |
| Denaturation Temperature: | 95 °C - | 30sec | 35-40 cycles |
| Annealing Temperature: | 55-62 °C - | 30-45 sec | |
| Extension Temperature: | 72 °C - | 1 min | |
| Final Extension Temperature: | 72°C - 1 min | | |
| Hold: | 4 °C - ∞ | | |

The nucleotide sequence of the primers used in this study: see Appendix 2.

### 2.2.6.1 DNA agarose gel electrophoresis

Following PCR the amplified products were resolved in 2% agarose gel in 0.5xTBE. 10,000 x diluted SyberSafe stain (Invitrogen) was used to visualize the DNA under UV image analyser. 12-20 µl of the sample was loaded into a gel and the electrophoresis was maintained at a constant voltage of 110V for 40-50 min in running buffer-0.5xTBE in horizontal gel electrophoresis system (Bio-Rad). In order to measure the molecular weight (MW) of the PCR product 1 µl of DNA ladder was loaded onto the gel (100bp DNA Ladder, BioLabs). Following electrophoresis the DNA bands were visualized under UV light and the photographs of the agarose gels were taken from the gel documentation system. In the present study

Glyceroaldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a housekeeping gene to normalise gene expression in RT-PCR.

## 2.2.7 Real Time PCR (qPCR) reaction

Quantitative analysis of mRNA expression was performed by real time PCR (qPCR), which used the intercalating dye SybrGreen during polymerase chain reaction. cDNA of samples were prepared as in section 2.2.5 A relative gene expression was analysed according to fold change method (Livak et al., 2001). Primer efficiency was calculated before qPCR reaction (Appendix 3). Only primers with an efficiency of 95-105% were used. SybrGreen master mix was purchased from BioRad and the manufacturer's protocol was followed. Primer sequences are described in Appendix 3. The reagents were prepared as follows:

- 1 µl of cDNA (10-25ng)
- 4 µl of Master Mix Buffer (which contains Taq polymerase and SYBRGreen)
- 1 µl of mixed primers (final concentration 10µM each)
- 2 µl water

The analysis was performed on the LightCycler 96 (Roche). The reactions were performed in triplicates for each analysed gene. Reactions employing SYBRGreen chemistry were subjected to melting curve analysis. Only one product was detected and analysed. Target gene expression was normalized using reference gene Beta-2 Microglobulin (B2M).

## 2.2.8 Protein purification

#### 2.2.8.1.1 Cell lysis for protein extraction

Confluent cells were washed twice with cold PBS. 400 µl protein lysis buffer was added to 75cm$^2$ cell culture flask (or 200µl per a well from 6-well plate). Cells were scraped, incubated for 30 min at 4$^0$C and centrifuged at 12,000g for 10 min at 4$^0$C in

order to get rid of all debris. The supernatant was collected and stored in -80$^0$C. The protein concentration was assessed using the Bradford Assay.

#### 2.2.8.1.2 Tissues lysis for protein extraction

Fatty tissue was collected and snap frozen in Liquid Nitrogen (LN2). 0.2-0.5 g was homogenized by mortar and pestle with the addition of liquid nitrogen (LN2). 300µl-500µl of Protein lysis buffer was added. The lysate was kept for 30min at 4$^0$C, vortexed several times and then centrifuged at 12,000g 10 min at 4$^0$C in order to get rid of all debris. The supernatant was collected and stored in -80$^0$C. The protein concentration was assessed using the Bradford Assay.

#### 2.2.8.1.3 Bradford Assay

Protein concentration from either cell culture or tissue lysate was analysed using the Bradford assay (BioRad). 1µl of lysate was diluted in 4µl of water. A protein standard curve was prepared by serial dilution of Bovine Serum Albumin (BSA) with a starting concentration 1µg/ml in water in a range from 1000-62.5 ng/ml. 5 µl of each diluted sample (1:5) and standard in triplicates was added to 250µl Bradford reagent in 96-well microplate and incubated for 5 minutes at RT. Absorbance was read at 595nm on microplate reader iMARK (Bio-Rad, Japan).

## 2.2.9 Western Blot

### 2.2.9.1 SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis)

SDS-PAGE was carried out on the Small Biorad System. In brief, 12% Acrylamide/Bis-Acrylamide gel was prepared using the following protocol. The separating gel was prepared by adding 9ml of water to 6 ml of acrylamide/bisacrylamide followed by 5ml of Separating buffer, 100µl Ammonium Persulphate (AMPER) and 10µl Tetramethylethylenediamine (TEMED). Gels were allowed to polymerise for 30-45 min. The stacking gel was prepared by adding 6.5 ml of distilled water, 1ml of acrylamide/bisacrylamide and 2.5ml of Stacking buffer followed by 50µl AMPER and 10µl TEMED. The gel was poured, the comb aligned and the gel was allowed to cool down and polymerise for the next 30min.

50 µg of samples were diluted (1:1) in Laemmli Denaturating Loading Buffer (which contains β-mercaptoethanol), incubated at $100^0$C for 5 min and then chilled on ice in order to denature proteins. Molecular marker and samples were loaded into the gel wells. The gel was run for about 30 min at 80V until samples had passed through the stacking gel and had reached the top of the separating gel. The voltage was then increased to 135V and electrophoresis continued for 1-2h.

### 2.2.9.2 Electrophoretic transfer

After electrophoresis gels were allowed to equilibrate for 10-15 min in Transfer Buffer. Polyvinyl (PVDV) membranes were soaked for 10s in 100% methanol. The transfer sandwich was prepared with using buffer soaked fiber pads and filter papers (Whatman).

The cassettes were assembled in BioRad TransferSystem filled with Transfer Buffer. The cooling unit was inserted in order to keep a cold temperature during the transfer. Proteins were transferred for 1 hour at 100 V.

#### 2.2.9.3 Blocking and antibody incubation

Following transfer, membranes were washed in PBS for 5 min in order to remove methanol traces and blocked in Odyssey Blocking buffer solution in PBS (1:1). Membranes were then incubated with appropriate primary antibody overnight at $4^0$C. After washing 3 times in PBST membranes were incubated with the appropriate fluorescent conjugated secondary antibody (Li-Cor) (diluted 1:10,000) for 1h at RT and then washed 3x in PBST (for antibody dilution see Appendix 4). An Odyssey Fc Imager was used for detection and quantification of bound fluorescent antibody. To test non-specific binding of secondary antibodies the whole protocol was carried out in the absence of primary antibody. No binding was detected.

### 2.2.10 Tissue explant culture

Articular cartilage, synovium and IPFP were harvested under sterile condition after TKR surgery. Tissue was cut into 1-3$mm^3$ fragments, weighed and washed intensively with sterile PBS before culturing for 1 day in Seeding Medium. Explants were cultivated for 3 days in serum-free medium. To analyse protein secretion, the medium was collected, spun at 500g for 5 min at RT to remove floating cells and debris. The remaining supernatant was stored in -$80^0$C.

### 2.2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used. For adiponectin detection, Human Total Adiponectin/Arcp30 Quantikine ELISA kit was used. Primary antibody pre-coated wells were incubated with standard or sample diluted in Diluent at room temperature for 2h, washed 4 times with Wash Buffer, before addition of 200µl of conjugate and incubation for 2h at RT. Plates were washed 4 times, incubated 20 min with 200 µl of substrate solution in the dark. The reaction was stopped by addition of stop solution and the results were recorded by absorbance reading at 450nm and 570nm for colour correction on microplate reader iMARK (Bio-Rad, Japan).

For MCP-1/CCL2 ELISA commercially available Peprotech Human MCP-1 Mini Elisa Development Kit was used. 100 µl of capture antibody at 0.25µg/ml in PBS was added to each ELISA plate well and incubated overnight at room temperature.

Next day the plate was washed 4 times using 300µl/well of PBST before incubation with 300µl Peprotech ELISA Block Buffer for 2 hours at room temperature. After blocking, the plate was washed 4 times with PBST and incubated with 100µl standard or sample diluted in Peprotech Diluent for 3 hours at room temperature followed by 4 washes with PBST. Detection was conducted by adding 100µl of Peprotech Detection Antibody conjugated with biotin at a concentration of 0.5µg/ml in Peprotech Diluent for 2 hours at room temperature. The plate was then washed 4 times with PBST before incubation with 100µl of Avidin-HRP diluted in Peprotech Diluent for 30min at room temperature. A final 5 washes with PBST were followed by addition of 100µl of ABTS Liquid Substrate and incubation at room temperature for colour development. The results were recorded by absorbance reading at 415nm and 650 nm for colour correction on microplate reader iMARK (Bio-Rad, Japan). The explant culture data were calculated by division of the concentration per wet weight of the tissue.

### 2.2.12 Immunofluorescent staining (IF)

Passage 0 chondrocytes were seeded at high density on coverslips in a 12-well plate and cultured for 10-14 days. Cells were washed in PBS and fixed in Cell Fixing Solution for 10 min at -20$^0$C, cells were then air dried, washed in PBS and blocked in Dako Blocking Solution for 30min at RT. Incubation with primary antibody was conducted overnight at 4$^0$C, followed by 3xPBST washing and incubation with secondary fluorescent dye-conjugated antibody for 1h at RT in the dark. Coverslips were washed three times with PBST then incubated for 10 min in DAPI solution (1:5000 in distilled water) at RT and analysed using a Fluorescent Microscope (Nikon E800) (For antibody dilution see Appendix 4).

### 2.2.13 Immunohistochemistry (IHC) of paraffin embedded sections

#### 2.2.13.1 Tissue collection and proceeding.

Explants from synovium, IPFP, cartilage and bone were collected after surgery and washed extensively in an antimicrobial solution (Anti/Anti). Tissues were then fixed

in 4% formaldehyde for 24 hours at RT. Following three washes with PBS all tissues except bone explants were then processed and embedded in paraffin by a Histology Service (Breakthrough Western General Hospital). Fixed bone samples were decalcified for up to 2 weeks in 10% EDTA (pH=7.4) with changing of the solution every 3-4 days before embedding. Paraffin blocks were cut into 5µm sections, which were deparaffinised and rehydrated sequentially using: HistoChoice (equivalent for Xylene) for 10min twice, 100% ethanol for 2 min twice, 80% ethanol for 2 min, 50% ethanol for 2 min and water for 30 seconds.

Antigen retrieval was performed to reverse epitope masking. Antigen retrieval was performed in a pressure cooker (Northware). Tissue sections were incubated in a pressure cooker in Citric buffer (pH=6.0 see 2.1.2 List of Solution) pre-warmed for 5 min in the microwave. After pressure release slides were left to cool for 25 min and then incubated in 0.04 M $H_2O_2$ solution for 10 min to block the endogenous peroxidase activity of tissues enzymes. Slides were then washed once in PBS for 5 min and blocked in Dako Blocking Solution for 30 min at RT.

Sections were incubated with appropriate primary antibody diluted in Dako Diluent overnight at $4^0$C with final dilution usually 1:100 (or otherwise as stated see Appendix 4). On the following day, slides were washed three times with PBST for 5 min, incubated with secondary antibody conjugated with Horseradish Peroxidase (HRP) for 1h at RT (dilution 1:1000). Sections were then washed three times for 5 min in PBST. Chromogenic substrate 3,3'-Diaminobenzidine (DAB) was used to develop colour. 30µl of DAB was added to 1ml of DAB Buffer. 100µl of the DAB reagent mixture was applied onto the section. Sections were stained 5-10min depending on colour development. Sections were then washed with distilled water and counterstained by incubation in a Harris Hematoxylin solution for 10 seconds, followed by 20 seconds tap water washing and incubation for 20 seconds in Scott's Tap Water (see 2.1.2 List of Solutions). Slides were then dehydrated in serial changes of ethanol and HistoChoice: 50% ethanol for 30 sec, 80% ethanol for 30 sec, 100% ethanol for 2 min twice and HistoChoice for 10min twice. Sections were then mounted in a DPX mounting medium.

#### 2.2.13.2 IHC Scoring

IHC stained cartilage sections were subject to analysis by blind researcher and confirmed by trained pathologist. The intensity of staining was scored as follows: 0-no staining up to 4-very high-intensity staining.

### 2.2.14 Flow cytometry analysis

#### 2.2.14.1 Tissue digestion

Tissues (synovium and IPFP) were harvested after TKR as previously described. The stromovascular fraction (SVF) from each tissue was obtained as follows: About 1-3 g of tissue was intensively washed with sterile PBS and minced finely with scissors and scalpel blade into 1-2 $mm^3$ pieces which were then incubated for 1.5 h at $37^0$C in 2 mg/ml collagenase I and II mixture in 0.5% BSA in Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium. After digestion, the solution was passed through a sterilised metal strainer and centrifuged at 300g for 5 min at RT. The pellet after centrifugation was re-suspended in PBS and passed through a 100 µm strainer (Fisher) and centrifuged at 300g for 5 min at RT before addition of 1ml Red Cells Lysis Buffer for 10-15 min. The cell suspension was then washed with PBS and centrifuged at 300g for 5 min. The remaining pellet was resuspended in PBS and passed through a 70 µm strainer (Fisher). Cells were then diluted in order to obtain a density of approximately 0.5-1 million cells per 1 ml and assessed with Immunofluorescent Flow Cytometry Staining.

#### 2.2.14.2 Immunofluorescent Flow Cytometry Staining

100 µl of cells were incubated with appropriate concentrations of specific antibody or antibody isotype control for 30 min at $4^0$C in the dark. The standard antibody dilution was 1:100. Cells were then washed twice with PBS and centrifuged at 300g at RT and analysed by Flow Cytometry (for antibody dilution see Appendix 4). Beckman-Coulter XL Flow Cytometer was used to obtain results. Minimum 50000 events were collected with using electronic Gate to exclude debris and dead cells so the data for a minimum of 10000 'live' events occurred in the gate (Live cells gate). Data for all events were collected. The example of gating strategy is shown in Appendix 5. The compensation to exclude overlap between fluorescent

channels for two and three colour staining was applied post-acquisition using single colour stained by FCS Express Flow Cytometry 5 software (Example in Appendix 5).

## 2.2.15 Micro-Computed Tomography (µCT) analysis of Femoral Condyles from OA patients

### 2.2.15.1 µCT imaging, reconstruction and analysis of Femoral Condyles of OA patients

Minimally or non OA-affected posterior lateral femoral condyles obtained after TKR was collected and stored in -20$^0$C until analysis (as previously described). The tissues were placed in Skyscan ® 1172 micro-CT scanner (SkyScan, Belgium) and bone microstructure was evaluated using following parameters: the beam set 70kV, 140µA, and a 6.71 µm pixel size with 0.5mm aluminum filter. Image reconstruction was conducted using to Skyscan ®NRecon software and analysed by Skyscan ®CTan software. For µCT parameters evaluation 3mm$^3$ VOI (voxel of interest) was chosen in the middle of the condyle below subchondral plate region as shown in Figure 7.2.

## 2.3 Statistical analysis

For semi-quantitative analysis of RT-PCR, the densitometry analysis was performed using Image J analysis software (NIH). The intensity of the bands taken from photographs was recorded by the software. For Western Blot data, semi-quantitative analysis of bands intensity was performed by Image Studio Lite Software v.5.0. The arbitrary values of the band intensities obtained by the software were used for mathematical calculation for the expression of the gene and protein of interest. The ratio of the values between the gene of interest and housekeeping gene was considered as a ratio of gene expression. The ratio of the values between the protein of interest and reference protein was considered as the ratio of protein expression. All statistical analysis was performed using GraphPad (Prism 6.0, USA). Differences in gene expression by RT-PCR, Real Time PCR, IHC scoring, semi-quantitative WB protein expression, ELISA protein secretion, Flow Cytometry analysis and µCT parameters were assessed for unpaired sample by Mann-Whitney U test and Wilcoxon matched paired test were used to compare linear variables between groups and Spearman's rho correlation was used to assess the association between linear variables p-value $< 0.05$ was considered to indicate statistical significance.

# Chapter 3: The differences in obesity-related markers expression in chondrocytes and cartilage from lean and obese OA patients

## 3.1 Introduction

The key molecular factors in OA and obesity interaction have not been fully described. Since obesity and osteoarthritis are multidimensional diseases, no single shared molecular marker has been identified. In fact, a large range of proteins has been postulated to play a role in a crosstalk between adipose tissue and cartilage (Conde et al., 2014; Sartori-Cintra et al., 2014; Conde et al., 2015). The purpose of this analysis was to screen possible gene and protein candidates, which may play an important role in adipose tissue and cartilage interaction.

Cartilage is very specific yet a very difficult tissue to analyse. Its three-dimensional structure which is mainly made up of the matrix with only a relatively small number of cells makes it very hard to investigate using standard molecular biology techniques including mRNA and protein isolation. Hence, the culture of chondrocyte monolayers is a widely used technique in gene and protein expression studies of cartilage biology (Owen et al., 2008; Zeggini et al., 2012), though this has limitations as it does not mimic the 3D cartilage environment it can be useful for preliminary examination. Chondrocytes isolated by cartilage digestion, kept at the lowest passage, maintain chondrogenic gene expression including Type II Collagen (COL2A1) and Aggrecan (ACAN) (Ono et al., 2013) and can be a potential model in OA studies (Marlovits et al., 2004). However, with further passaging these chondrocytes loose chondrogenic potential (de-differentiate) and become fibroblasts (Caron et al., 2012).

In the present study, genes and protein candidates, which could play an important role in obesity's contribution to OA were screened. Chondrocyte gene expression of the main adipokines, including adiponectin (ADIPOQ), leptin (LEP), visfatin

(NAMPT), chemerin (TIG2), lipocalin 2 (LCN2) and some of their reported receptors such as ADIPOR1, ADIPOR2 (for adiponectin), LEPR (for leptin) and CMKLR1 (chemokine-like receptor 1) (for chemerin) were investigated. Additionally, the gene expression of two important nuclear factors which play a role in adipogenesis: namely PPARγ (Peroxisome Proliferator-Activated Receptor γ) and C/EBPβ (CAAT/Enhancer-binding protein β) was analysed. Also, reported pro-inflammatory chemokine CCL2/MCP-1 and recently described genes such as FTO (fat-mass associated gene) connected with obesity and OA, TN-C (tenascin C) postulated to be up-regulated during cartilage damage (Nakoshi et al., 2010), surface markers such as VCAM1 (Vascular Cell Adhesion Molecule 1) which is an important adhesion molecule during lymphocytes migration to the site of inflammation(Conde et al., 2012) and TLR4 (Toll-like receptor 4) which is a key DAMP receptor and is postulated to play key role in both obesity and OA were investigated (Jialal et al., 2014; Gomez et al., 2015).

At the protein level, 3 main candidates were investigated: visfatin, PPARγ, and CCL2.

### 3.1.1 Aims

One of the hypotheses of the present study was that the molecular pathways involved in osteoarthritis are different in obese and lean patients. Therefore, differences in expression of obesity markers were investigated in cartilage and chondrocytes of patients undergoing knee replacement surgery. The main aim of this part of the study was:

1. To screen for gene expression of possible candidates for molecular players in the pathogenesis of the OA/obesity interaction.
2. To find if there was a differential expression of obesity-related biomarkers in chondrocytes and cartilage from OA patients between obese and lean individuals.

## 3.2 Chondrocytes Gene Expression Analysis

### 3.2.1 Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis

#### 3.2.1.1 Chondrogenic potential of chondrocytes in monolayer cell culture

The human chondrocytes obtained after Collagenase II digestions were cultured in passage 0 up to 14 days. Before assessing the expression of obesity related genes in isolated chondrocytes RT-PCR for chondrogenic genes ACAN (Aggrecan) and COL2A1 (collagen type II) was used to confirm they retained chondrogenic potential (see Figure 3.1).

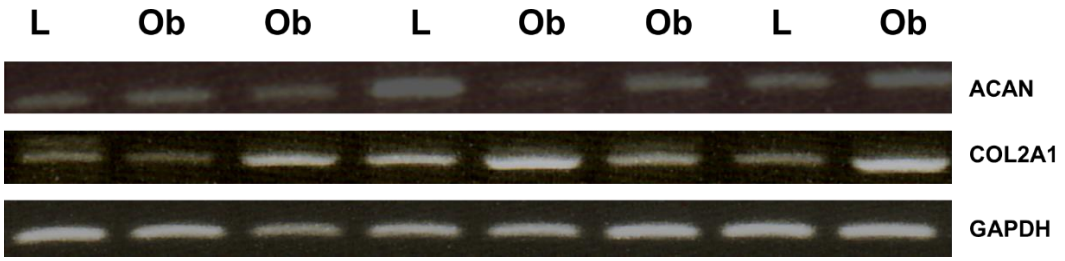



**Figure 3. 1 Expression of the chondrogenic marker in chondrocytes isolated from OA cartilage.** Chondrocytes from monolayer cell culture at passage 0 maintain chondrogenic potential. Expression of chondrogenic genes: ACAN and COL2A1 next to reference gene GAPDH were presented in 8 samples (Representative RT-PCR result for all analysed samples). L-lean patient, Ob- Obese patient.

RT-PCR confirmed the maintenance of chondrogenic bio-markers (Figure 3.1) by isolated chondrocytes. Cultured chondrocytes do not de-differentiate.

### 3.2.1.2 Expression of obesity/OA-related genes by chondrocytes in monolayer cell culture

The expression of obesity-related genes by chondrocytes was ascertained after 10-14 days in monolayer cell culture using RT-PCR (see Figure 3.2).

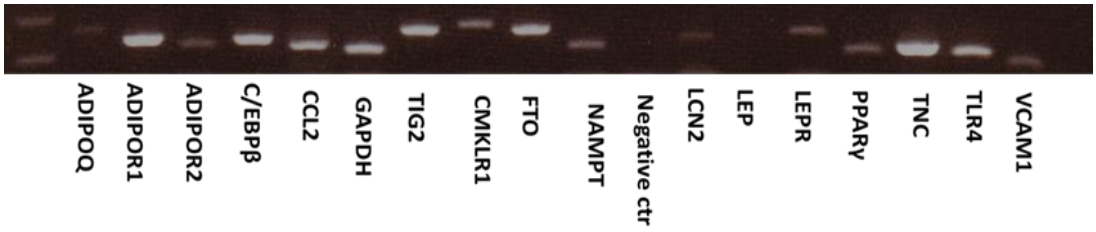



**Figure 3. 2 Representative expression of OA/obesity-related genes in chondrocytes in monolayer culture.**
Expression of main adipokines ADIPOQ (adiponectin), LEP (leptin), TIG2 (chemerin), NAMPT (visfatin), LCN2 (Lipocalin 2), their receptors (ADIPOR1, ADIPOR2, CMKLR1, LEPR), chemokine monocyte-chemotactic protein 1 (CCL2), nuclear factors (C/EBPβ, PPARγ) and other inflammation-related proteins: FTO, TN-C, TLR4 and VCAM1 was confirmed by RT-PCR in monolayer chondrocyte cell culture maintained at passage 0. A representative RT-PCR result is shown.

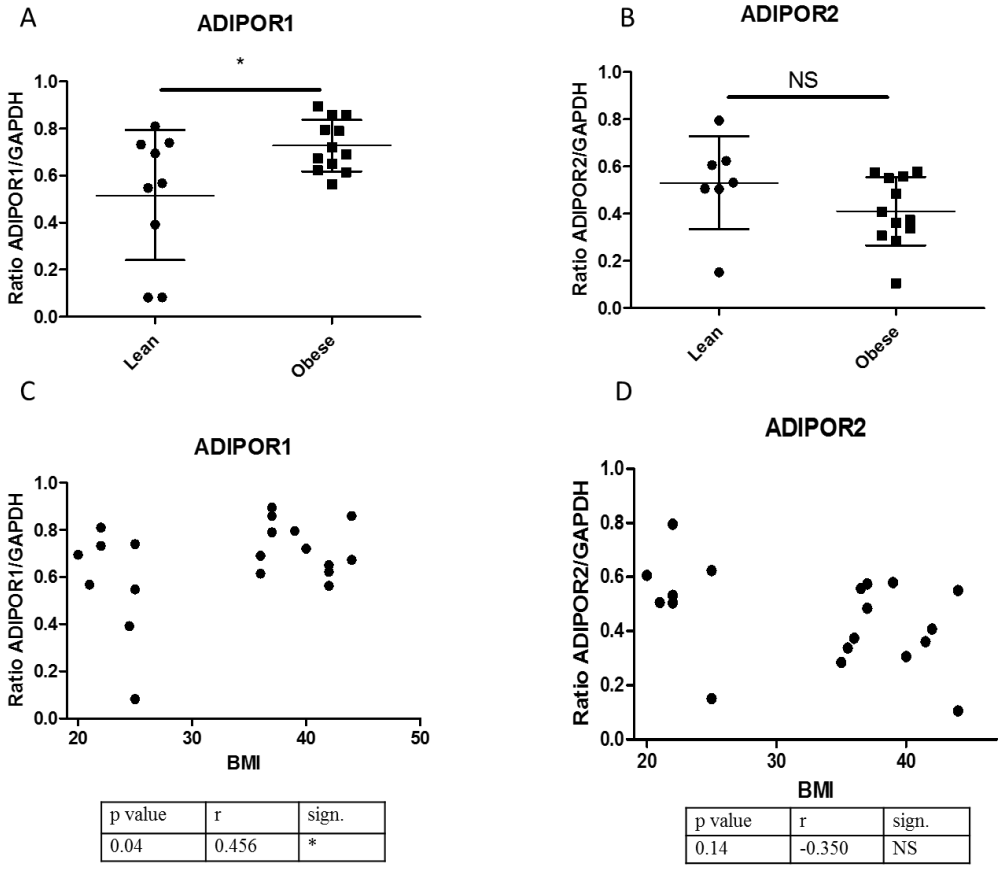
Adiponectin (ADIPOQ) and leptin (LEP) expression was very low or undetectable, however, expression of their receptors ADIPOR1 and ADIPOR2 and LEPR was confirmed in cultured chondrocytes. mRNA for two adipokine genes; lipocalin 2 (LCN2) and visfatin (NAMPT) was also detected. Chemerin (TIG2) and its receptor CMKLR1 (chemokine-like receptor 1) were expressed in chondrocytes as well.

Chemokine Monocyte-Chemotactic Protein 1 (MCP-1) also called chemokine (C-C) ligand 2 (CCL2) and Fat mass associated gene (FTO), recently shown to correlate with OA progression, were also detected in OA chondrocytes. Transcription factors including CAAT Enhancer-Binding protein β (C/EBPβ) and Peroxisome Proliferator-Activated Receptor γ (PPARγ) were also expressed in the human chondrocytes.

Tenascin-C (TN-C) and two pro-inflammatory surface markers: Toll-Like Receptor 4 (TLR4) and Vascular Cell Adhesion Molecule 1 (VCAM1), were also present in the cultured human chondrocytes (Figure 3.2).

### 3.2.1.3 Differences in gene expression in monolayer cell culture of chondrocytes from lean and obese patients with OA

Chondrocytes were isolated from OA patients and classified into lean and obese groups on the basis of their BMI index. Patients with a BMI below or equal to 25 were described as LEAN while patients with BMI equal to or above 35 were described as OBESE. Figures 3.3-3.6 show graphs describing the differential gene expression between the two groups.




| p value | r | sign. |
|---|---|---|
| 0.04 | 0.456 | * |

| p value | r | sign. |
|---|---|---|
| 0.14 | -0.350 | NS |

**Figure 3. 3 Semi-quantitative PCR analysis of Adiponectin Receptors (ADIPOR1 and ADIPOR2) in chondrocytes obtained from Lean and Obese patient with OA.**
RT-PCR semi-quantitative analysis of ADIPOR1 and ADIPOR2 expression in OA patients. Two groups termed "Lean" n=8 for ADIPOR1 n=7 for ADIPOR2 and "Obese" n=12 were divided according to BMI. GAPDH was used as a reference gene. The distribution of data, mean ratio values (horizontal line) for each group and standard deviation (vertical line) (SD) bars are shown in A,B. The correlation between ratio values and BMI score are shown in C,D. p values < 0.05 were considered significant and indicated *.

Chondrocytes isolated from both groups of patients expressed adiponectin receptors ADIPOR1 and ADIPOR2. Expression of ADIPOR1 was significantly higher in chondrocytes from obese as compared to lean patients whereas there was no

difference in ADIPOR2 expression. ADIPOR1 expression correlated significantly with BMI.

The difference between ADIPOR1 and ADIPOR2 expression in chondrocytes was analysed further (see Figure.3.4)

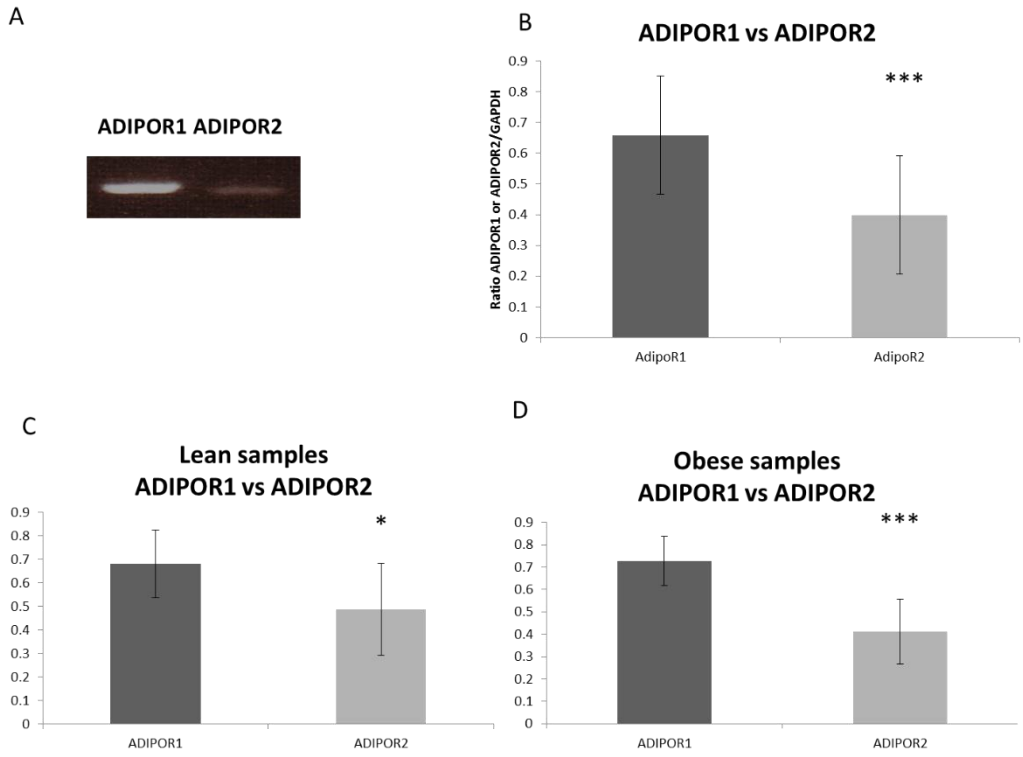



**Figure 3. 4 Differences in expression of adiponectin receptors, ADIPOR1 vs ADIPOR2 in OA chondrocytes.**
RT-PCR semi-quantitative analysis of ADIPOR1 versus ADIPOR2 expression in OA chondrocytes. Representative RT-PCR results shown in (A). ADIPOR1 vs. ADIPOR2 expression in all obtained chondrocytes is shown in (B). Lean and Obese patients' chondrocytes n=8 ADIPOR1 vs ADIPOR2 expression is shown in (C, D). GAPDH was used as a reference gene. The mean ratio values for each group and standard deviation (SD) bars are shown in (B-D). The difference was analysed by Wilcoxon matched-pairs signed rank test. p-value <0.05 is indicated as *, p value<0.001 as ***.

Conducting a semi-quantitative analysis of ADIPOR1 versus ADIPOR2 expression within chondrocyte samples from the same donor, showed that regardless of the BMI ADIPOR2 expression was significantly lower ($p<0.001$) than that of ADIPOR1. Comparing the expression of adiponectin receptors in chondrocytes from lean or obese patients showed that expression of ADIPOR1 was still significantly higher than ADIPOR2 in both groups ($p<0.05$ and $p<0.001$ respectively).

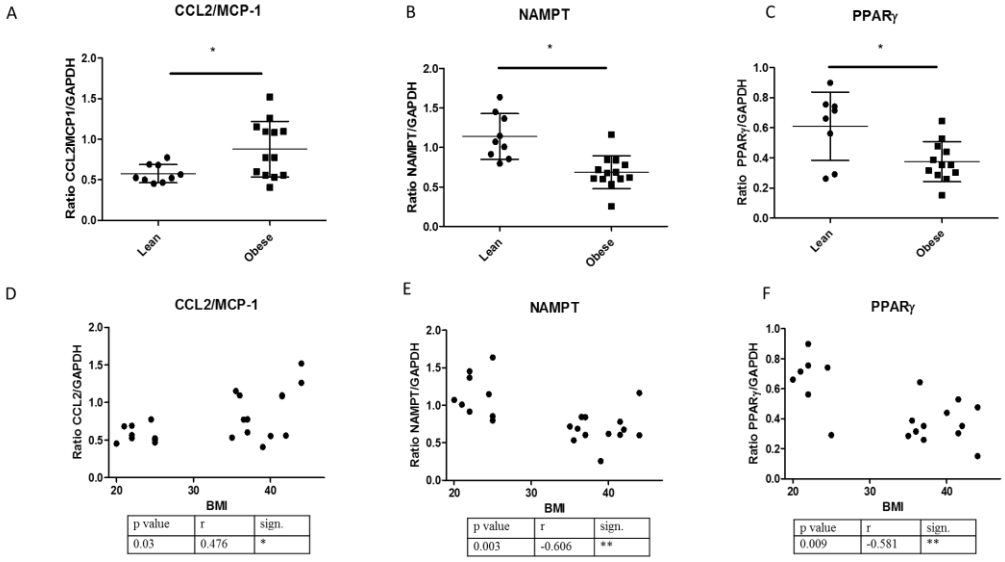



**Figure 3. 5 Semi-quantitative PCR analysis of CCL2/MCP1, NAMPT (visfatin) and PPARγ in chondrocytes obtained from Lean and Obese patients with OA**
Semi-quantitative densitometry RT-PCR analysis of CCL2/MCP1, NAMPT (visfatin) and PPARγ expression in chondrocytes obtained from lean and obese patients. Two groups termed "Lean" n=8 for CCL2/MCP-1 and PPARγ n=9 for NAMPT and "Obese" n=12 for PPARγ and n=13 for CCL"/MCP-1 and NAMPT were divided according to BMI. GAPDH was used as a reference gene. The sample distribution, the mean ratio values (horizontal lane) for each group and standard deviation (vertical lane) (SD) are presented (A-C). The correlation between ratio values and BMI score is shown (D-F). p values < 0.05 were considered significant.

Semi-quantitative analysis by RT-PCR showed that expression of the CCL2/MCP-1 gene was significantly higher in the obese as compared to the lean group. Whilst, NAMPT (visfatin) and PPARγ expression was significantly lower in chondrocytes from obese patients in comparison to lean ones. The correlation with BMI score for the ratio of CCL2/MCP-1/GAPDH expression was positive and that of NAMPT/GAPDH and PPARγ/GAPDH negative (Figure 3.5).

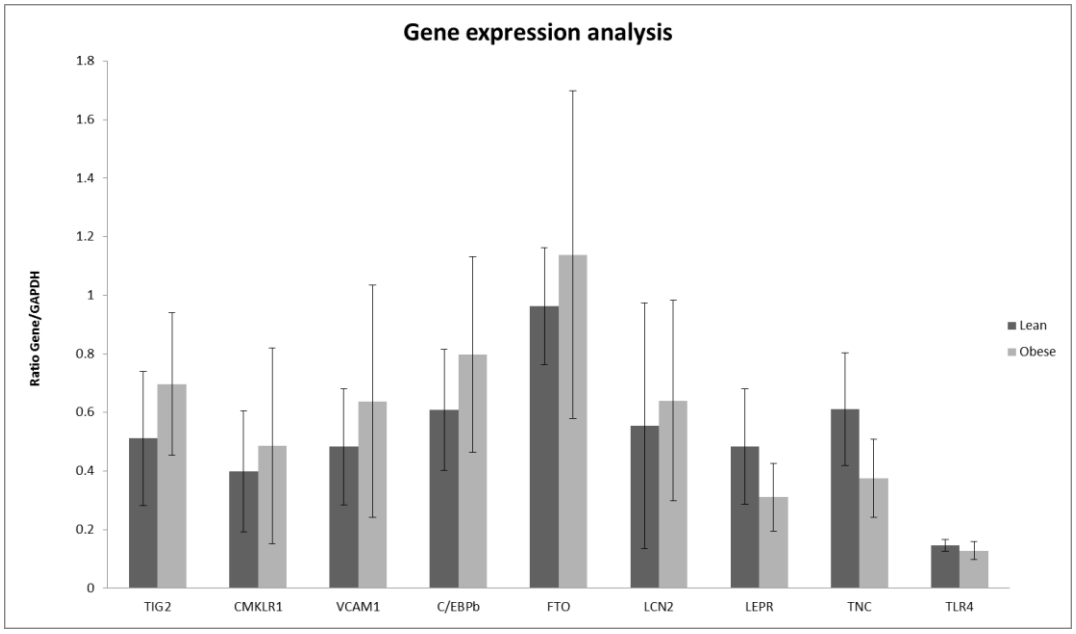



**Figure 3. 6 Semi-quantitative PCR analysis of genes in chondrocytes obtained from different OA patients.**
Two groups termed "Lean" n=8 and "Obese" n=12 were defined according to BMI. GAPDH was used as a reference gene. The mean ratio values for each group and standard deviation (SD) are shown.

The remaining genes that were investigated did not show any significant differences between lean and obese chondrocytes.

### 3.2.2 Quantitative Real Time (qRT) PCR analysis of obesity-related genes in monolayer chondrocytes.

Genes are shown by RT-PCR to have significantly different expression in chondrocytes between lean and obese patients were analysed using quantitative Real-Time qPCR. cDNA from a new set of patients for each group (lean/obese) were analysed in triplicate and gene expression was normalized to housekeeping gene beta-2microglobulin (B2M).

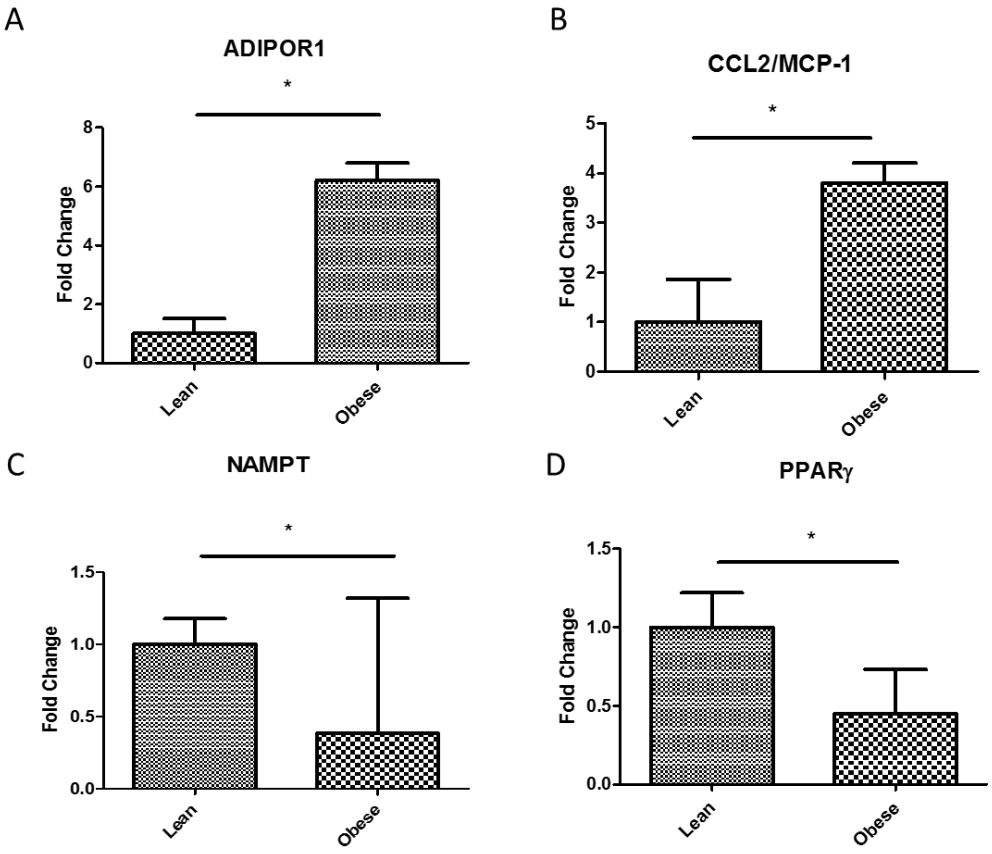



**Figure 3. 7 Real Time qPCR analysis of ADIPOR1, CCL2/MCP-1, NAMPT and PPARγ expression in chondrocytes from lean and obese OA patients.**
Quantitative PCR analysis of ADIPOR1 (A), MCP-1/CCL2 (B), NAMPT (C) and PPARγ (D) expression in chondrocytes obtained from different OA patients (A-D). Two groups termed "Lean" n=10 and "Obese" n=10 were defined according to BMI as before. B2M was used to normalise gene expression. The -ΔΔCT method was used. The mean value of Lean Group was used as a calibrator to investigate fold change of gene expression. The ratio values of each group and SEM bars are shown. p values < 0.05 were considered significant and indicated as *.

Quantitative Real-time PCR data confirmed the semi-quantitative RT-PCR results. The expression of ADIPOR1 and CCL2/MCP-1 was significantly higher, whilst expression of NAMPT (visfatin) and PPARγ was significantly lower in obese as compared to lean patients. ADIPOR2 gene expression was also investigated by Real Time PCR and there was no difference between Lean and Obese OA patients. However, there was a significantly higher expression of ADIPOR1 than ADIPOR2 confirming obtained RT-PCR data (data are shown in Appendix 6).

## 3.3 Differences in protein expression in Cartilage and Chondrocytes from OA patients

### 3.3.1 Visfatin protein expression in cartilage and chondrocytes

To determine whether the differences in gene expression were also seen at the protein level explanted tissue was subjected to immunohistochemistry (IHC) while monolayer chondrocytes to an immunofluorescence (IF) technique using specific anti visfatin antibody.

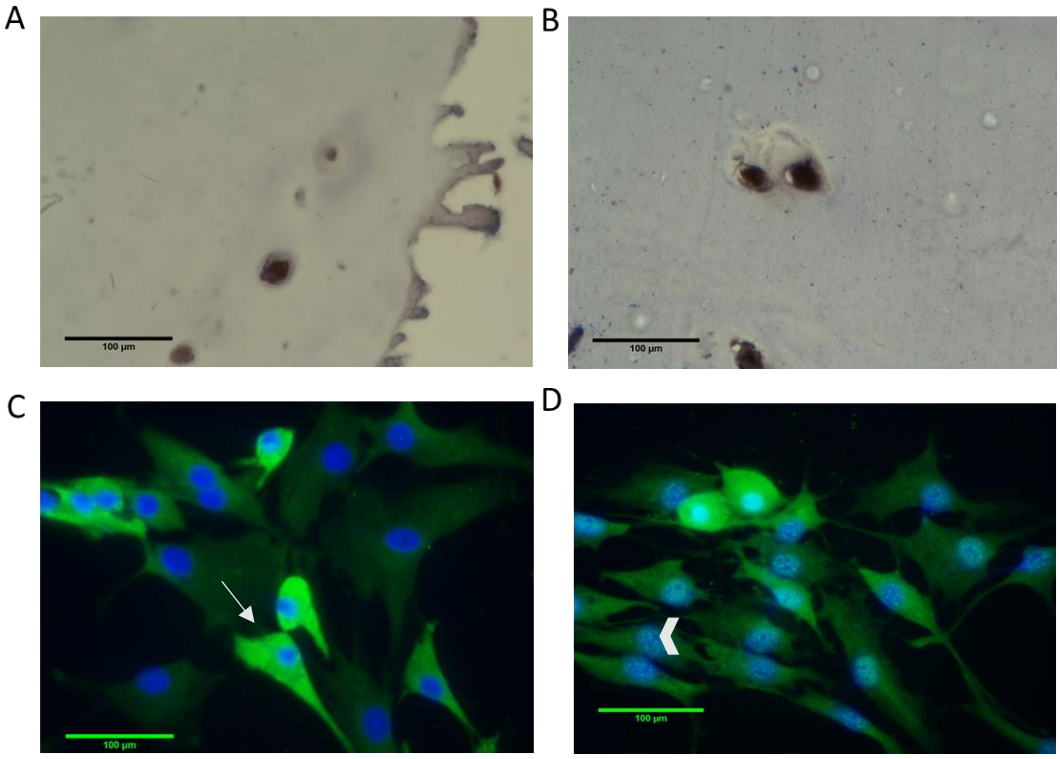



**Figure 3. 8 Visfatin expression in OA Cartilage and chondrocytes.**
Representative IHC and IF staining of visfatin protein expression in OA Cartilage (Brown colour) (A,B) and Chondrocytes (Green colour) (C,D). Both nuclear (arrowheads) and cytoplasmic (arrows) localisation of visfatin can be detected in monolayer cell culture. Isotype control staining are presented in Appendix 7. Blue colour indicates nuclear staining (using DAPI staining).

Visfatin was detected in cartilage, and in monolayer chondrocytes culture. IF staining show both nuclear and cytoplasmic locations within chondrocytes in monolayer cell culture (see Figure 3.8).

Blinded scoring of visfatin expression in cartilage explants (0-4) was conducted on IHC stained sections (Figure 3.9).

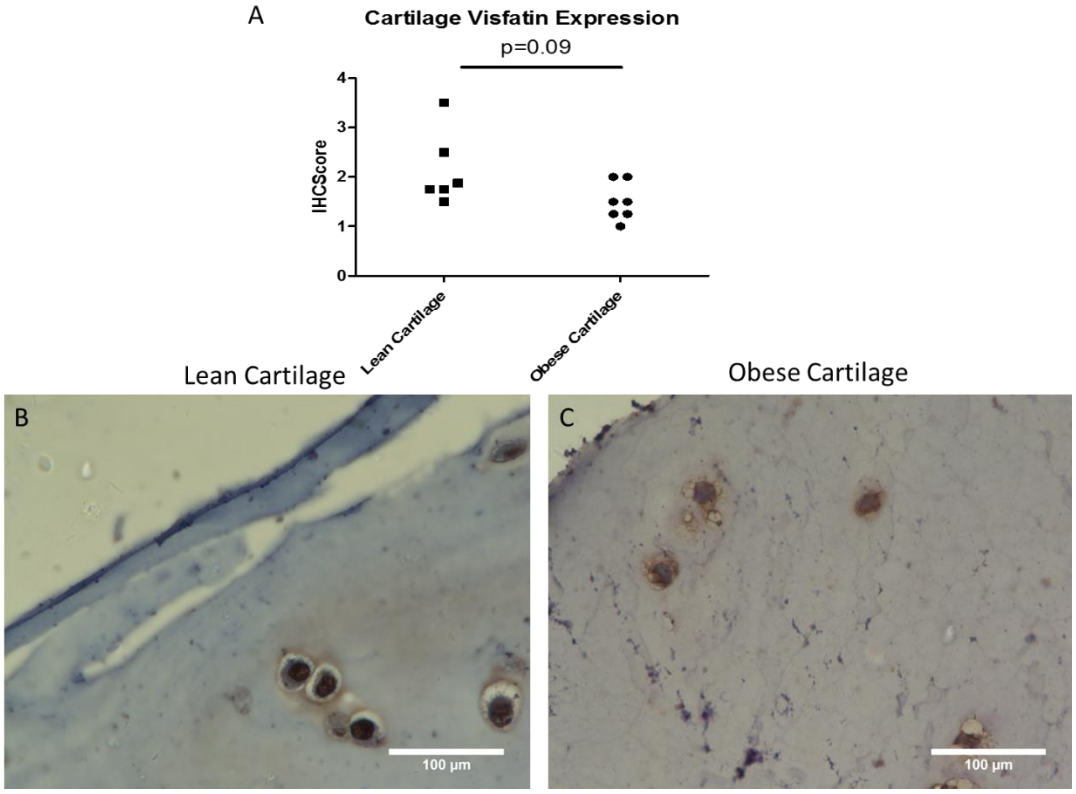



**Figure 3. 9 Visfatin expression in lean and obese OA patients' cartilage.**
The difference in visfatin protein expression (assessed using blinded IHC scoring) in cartilage from lean (n=6) and obese (n=7) patients (A). Data is presented as the dot plot. Representative IHC staining showing the difference in visfatin expression (Brown colour) between the cartilage from Lean (B) and Obese (C) patients.

IHC scoring showed that visfatin protein expression in cartilage sections trended to be higher in lean as compared to obese patients. The semi-quantitative differences in

Visfatin protein expression were assessed further by Western Blot (Figure 3.10).

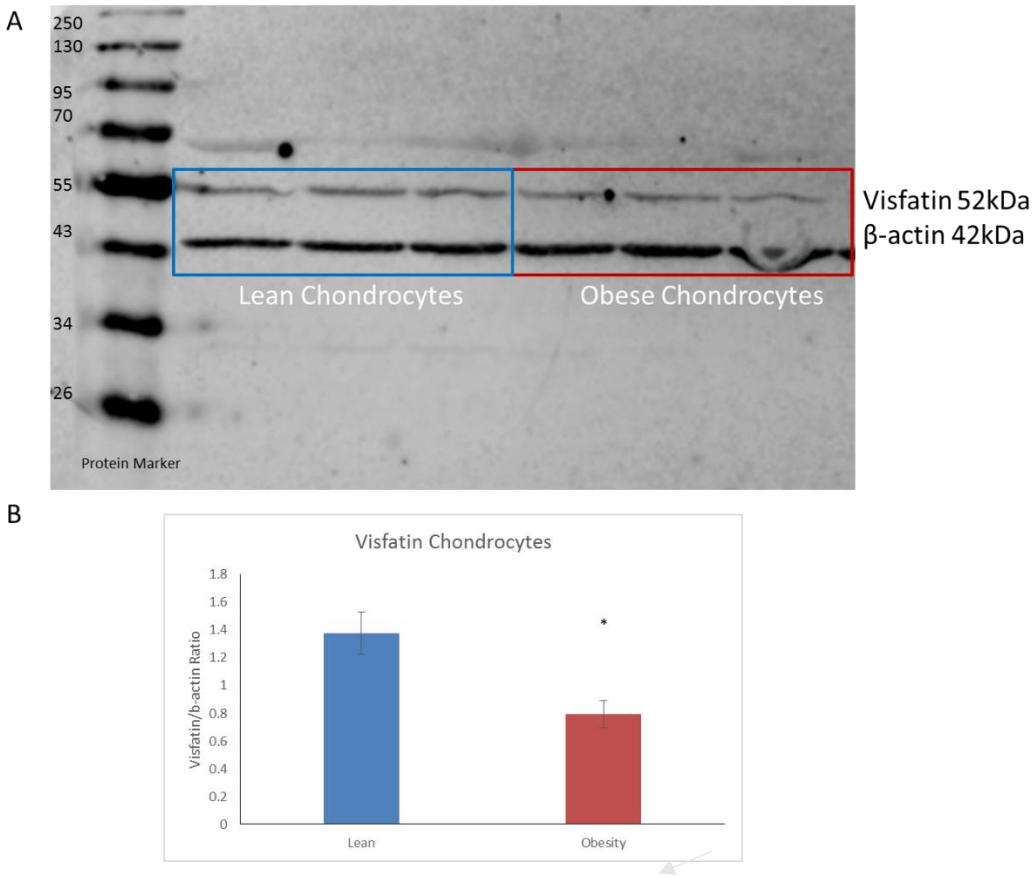



**Figure 3. 10 Visfatin expression in monolayer chondrocyte cell culture analysed by Western Blot technique.**
The results showed differences in visfatin expression between chondrocytes from lean and obese OA patients. Representative Western Blot results shown in A n=3 for each group. Semi-quantitative densitometry analysis of visfatin/B-actin ratios (n=5 for each group) (B). Data are presented as mean ratio ± SD. Statistically, significant differences between lean and obese group are indicated as * (*$p<0.05$).

WB analysis showed that chondrocyte visfatin expression in obese OA patients was significantly lower than in lean OA patients. Confirming previous RT-PCR and qPCR data that visfatin expression was significantly lower in chondrocytes from obese in comparison to the lean OA patients.

### 3.3.2 PPARγ protein expression in cartilage and chondrocytes

The expression of PPARγ protein was assessed using immunohistochemistry and immunofluorescence methods (Figure 3.11).

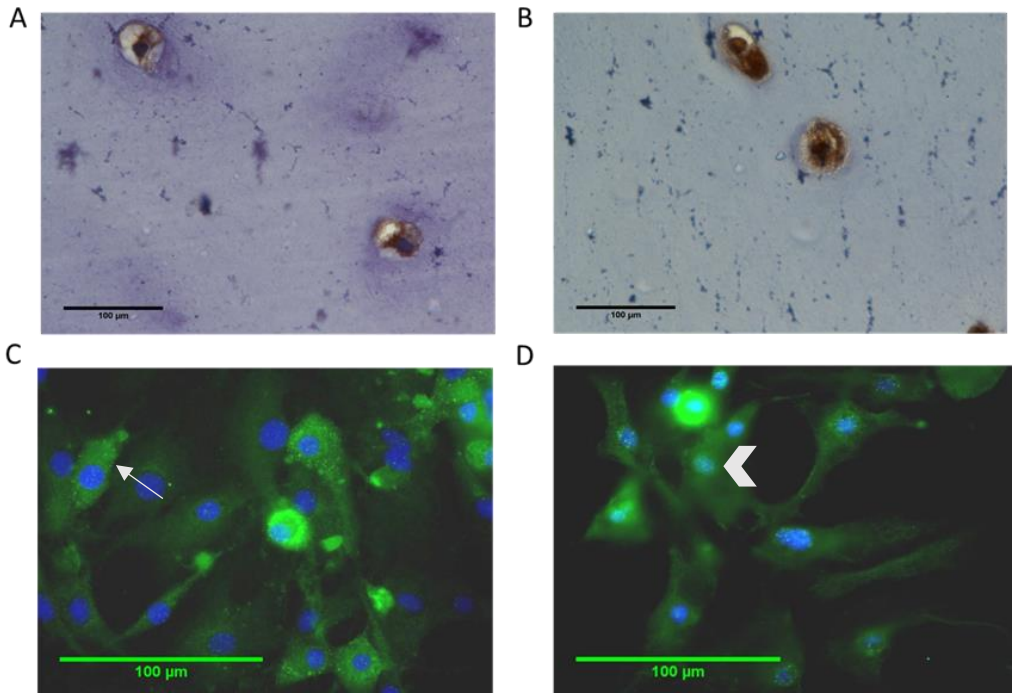



**Figure 3. 11 PPARγ expressions in OA Cartilage and chondrocytes.**
Representative IHC staining of PPARγ protein expression in OA Cartilage (Brown colour) (A,B) and in chondrocytes (green colour) (C,D). Both nuclear (arrowhead) and cytoplasmic (arrow) localisation of PPARγ were detected in monolayer cell culture. Isotype control staining are presented in Appendix 7. Blue colour indicates nuclear staining (using DAPI staining).

PPARγ was detected in cartilage, and in monolayer chondrocytes culture. IF staining show both nuclear and cytoplasmic locations within chondrocytes in monolayer cell culture (see Figure 3.8).

Blinded scoring of PPARγ expression in cartilage (0-4) was conducted on IHC stained sections (see Figure 3.12).

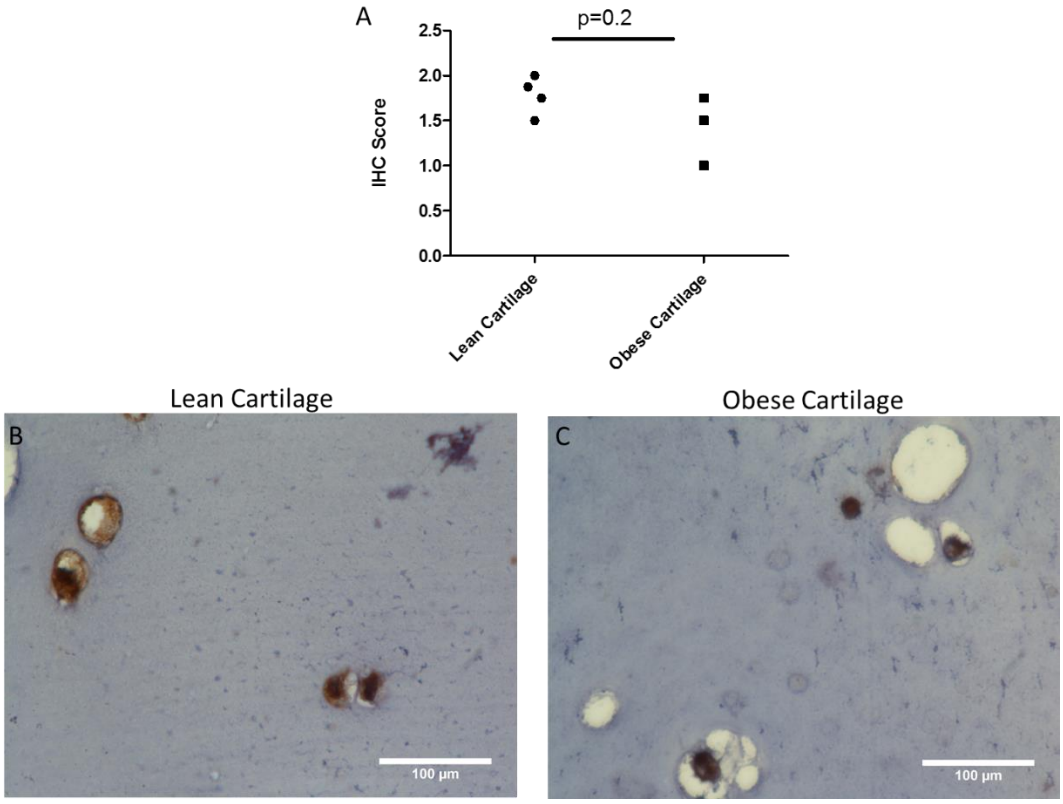



**Figure 3. 12 PPARγ expression in OA cartilage of lean and obese patients.**
Blinded scoring of difference in PPARγ expression in cartilage from lean (n=4) and obese (n=3) patients (A). Data are presented as the dot plot. Representative sections from lean (B-D) and obese (E-G) patients showing differences in PPARγ expression (Brown colour) in cartilage.

IHC scoring showed that staining of cartilage sections was consistent with PPARγ expression trended to be lower in obese cartilage as compared to lean, however, the difference was not significant. Possibly the immunohistochemical technique was not sensitive enough to detect a difference between the cartilage from lean and obese patients.

Assessing the difference between PPARγ protein expression in chondrocytes from lean and obese groups semi-quantitatively by Western Blot analysis (Figure 3.13).

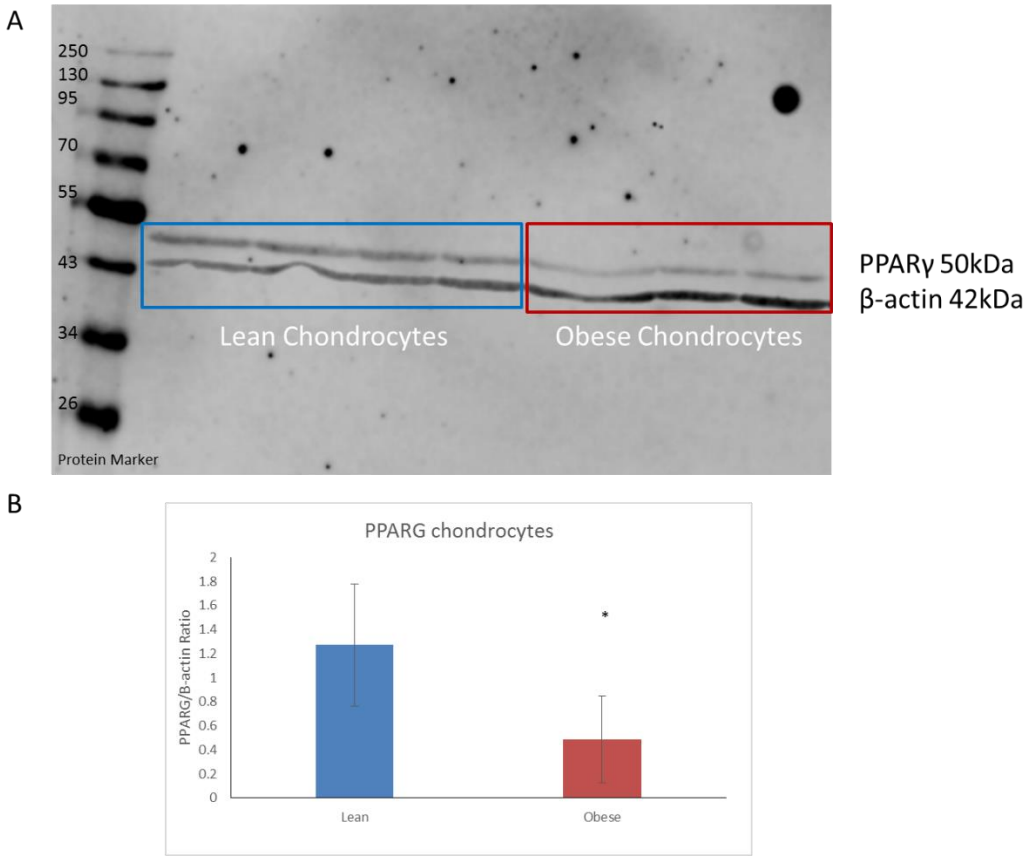



**Figure 3. 13 PPARγ expression in monolayer chondrocyte cell culture analysed by Western Blot technique.**

The results showed the difference in PPARγ expression between chondrocytes from lean and obese OA patients. Representative Western Blot result for n=3 for each group shown (A). Semi-quantitative densitometry analysis of PPARγ/B-actin ratios (n=5) (B). Data are presented as the mean ratio ± SD. Statistically significant differences between lean and obese groups are indicated as * (*p<0.05).

Semi-quantitative densitometry analysis suggested that the protein expression of PPARγ was significantly lower in chondrocytes from obese in comparison to lean OA patients concurring with the previously obtained PCR data.

### 3.3.3 CCL2 protein production by cartilage and chondrocytes

CCL2 protein production by chondrocytes and cartilage explants was analysed by Enzyme-Linked ImmunoAssay (ELISA). Confluent chondrocytes were serum starved for one day and then incubated in Serum Free media for one day. Cartilage explants were also serum starved for one day and incubated in serum-free medium for 3 days. The supernatant was then collected and analysed using a commercially available ELISA kit (MCP-1 Peprotech Mini Elisa kit) (Figure 3.14).

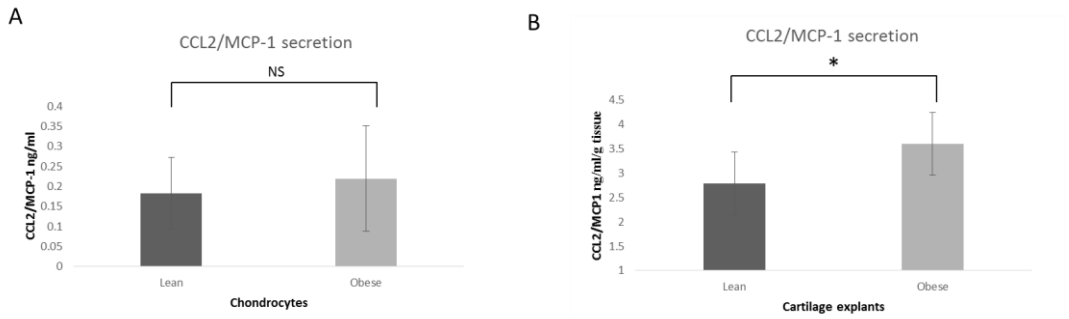



**Figure 3. 14 MCP-1/CCL2 secretion by OA Chondrocytes in cell culture and Cartilage explants**
The quantitative ELISA of supernatant from chondrocytes and cartilage explants (n=5 for each group) from lean and obese patients show that CCL2 was produced by chondrocytes and cartilage explants. Data are presented as mean ± SD. Statistically significant differences between lean and obese group are indicated as * (*$p<0.05$). NS- not significantly different.

Results from monolayer cultures (Figure 3.14) showed that OA chondrocytes from both groups were able to produce MCP-1/CCL2, though there was no significant difference between groups. However, cartilage explants from obese patients produced significantly more MCP-1/CCL2 than was found in the media collected from those of lean patients.

## 3.4 Conclusions and Summary

In conclusion, the data obtained revealed a number of important points:

1) Chondrocytes from patients with OA express a number of obesity-related genes including TIG2 (chemerin), NAMPT (visfatin), LCN2 (Lipocalin 2), adipokines receptors (ADIPOR1, ADIPOR2, LEPR, CMKLR1), monocyte-chemotactic protein 1 (CCL2/MCP-1), nuclear factors (C/EBPβ, PPARγ) and other inflammation-related proteins such as FTO, TN-C, TLR4 and VCAM1.
2) Chondrocytes from patients with OA express higher levels of adiponectin receptor ADIPOR1 than ADIPOR2.
3) Chondrocytes at monolayer cell culture from lean and obese patients with OA differ in expression of several genes connected with the pathogenesis of obesity, including ADIPOR1, NAMPT (visfatin), PPARγ and CCL2. Chondrocytes from obese OA patients express significantly lower NAMPT (visfatin) and PPARγ and higher ADIPOR1 and CCL2/MCP-1 gene in comparison to lean.
4) The protein level of visfatin, PPARγ and CCL2 is different between Lean and Obese Subject with OA in chondrocytes. Chondrocytes from obese OA patients produce significantly lower NAMPT and PPARγ and higher CCL2/MCP-1 protein.
5) CCL2 is secreted by both chondrocytes and cartilage explants. Cartilage explants from obese OA patients secrete significantly higher levels of CCL2.

All of the above data could help to explain the correlation between obesity and OA. The genes and proteins investigated in this study suggest different molecular pathways in the cartilage from obese and lean individuals. In particular, differences in PPARγ, visfatin, ADIPOR1 and CCL-2 expression in chondrocytes were found and need to be investigated further.

# Chapter 4: The differences in obesity-related markers expression in synovium from lean and obese OA patients

## 4.1 Introduction

The synovium is an integral component of the joint and is considered to play an important role in the pathogenesis of many arthritic diseases. The majority of studies focus on the synovial surface membrane layer, rather than the underlying fatty/fibrous layer of the synovium, and its contribution to more pro-inflammatory types of arthritis, including Rheumatoid Arthritis or Psoriatic Arthritis (Kruithof et al., 2005; Cañete et al., 2015). In these forms of arthritis, the synovial membrane is inflamed, thickened and oedematous. The hypothesis of the present study is that in OA synovium where the synovial membrane layer normally occupies a smaller percentage of the whole tissue, its other components including the supporting fat and fibrous tissue may play an important role in the pathogenesis of the disease.

Several proteins produced by adipose tissue are considered to be potent players in OA progression (Lubbeke et al., 2013). Previous studies suggest that the concentration of some molecules whose production in obese and lean individuals in OA is different (Rai et al., 2014). While most previous research has focused on protein content in serum and/or synovial fluid from OA patients, the purpose of this study is to investigate the role of local tissues within the knee joint in the production of obesity-related markers.

In this study, the contribution of synovium to the pathogenesis of OA was investigated. Expression of genes and proteins, which could play an important role in the involvement of obesity in OA were assayed. The gene expression in synovium of the main adipokines including adiponectin (ADIPOQ), leptin (LEP), visfatin (NAMPT), chemerin (TIG2), lipocalin 2 (LCN2) in addition to some of their receptors including ADIPOR1, ADIPOR2, LEPR, and CMKLR1 was assessed. Additionally, two important nuclear factors, namely PPARγ (Peroxisome

Proliferator-Activated Receptor γ) and C/EBPβ (CAAT/Enhancer-binding protein β) were analysed. Furthermore, inflammatory mediators, CCL2/MCP-1 a chemokine, the surface markers VCAM1 (Vascular Cell Adhesion Molecule 1) and TLR4 (Toll-like receptor 4) were also screened. At the protein level, six main candidates were investigated: adiponectin, visfatin, PPARγ, CCL2, VCAM-1 and TLR4.

The difference in gene and protein expression between lean and obese subjects with OA was assessed in order to explain the contribution of obesity to OA pathogenesis.

### 4.1.1 Aims

The main goal of the presented study was to compare differences in obesity markers in the synovium of patients undergoing knee replacement surgery.

Specifically, the aims were:

1. To screen the synovium for expression of possible obesity-related genes and proteins postulated to be involved in the pathogenesis of OA.
2. To determine if there was a differential expression of obesity-related biomarkers in synovium between obese and lean individuals with OA using different molecular biology techniques.

## 4.2 Differences in Synovial gene expression

Synovial biopsies were harvested from the knee joints of patients undergoing TKR (Total Knee Replacement) surgery. Patients were divided into two groups as previously described, i.e. Lean (BMI < 25) and Obese (BMI > 35). Each biopsy consisted of the synovial membrane layer and the supporting fatty/fibrous tissue The histological analysis did not show any correlation in the synovitis score between the obese and lean group (Representative H&E histology is included in Appendix 8).

### 4.2.1 Semi-quantitative RT-PCR analysis

A semi-quantitative RT-PCR technique was used to screen for candidate obesity markers to determine if there was a difference in gene expression in the synovium from lean and obese patients (Figures 4.1-4.3).

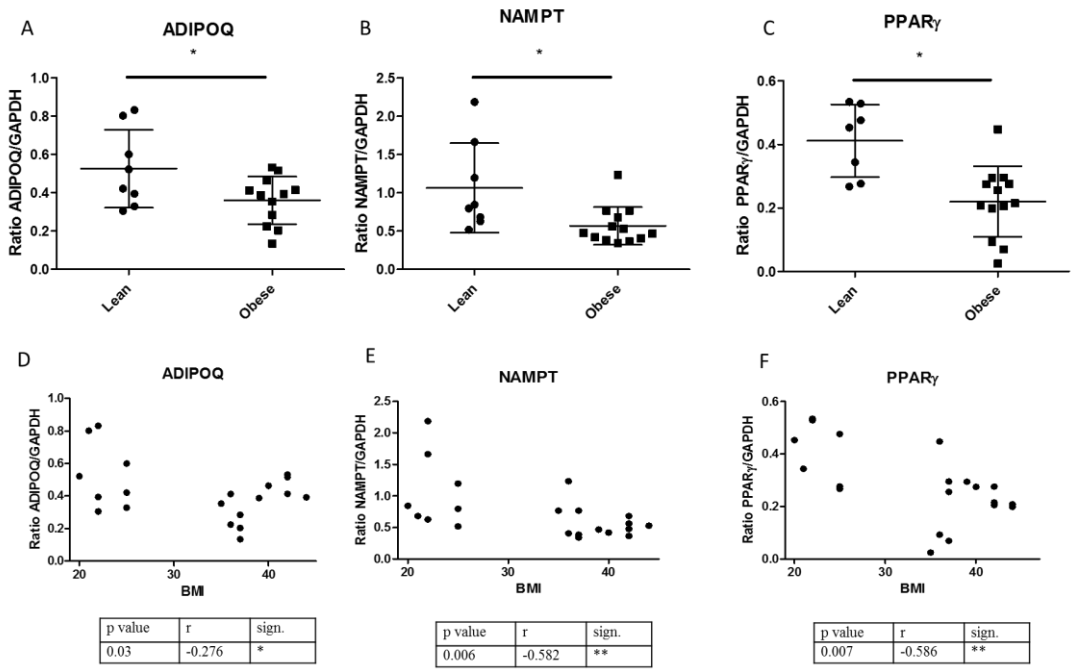



**Figure 4. 1 Semi-quantitative PCR analysis of ADIPOQ, NAMPT(visfatin) and PPARγ genes in synovium explants obtained from OA patient.**
RT-PCR semi-quantitative analysis of adiponectin (ADIPOQ), visfatin (NAMPT) and PPARγ genes expression in synovium from OA patients. Two groups termed "Lean" (n=8 for ADIPOQ and NAMPT, n=7 for PPARγ) and "Obese" (n=12 for ADIPOQ, n=13 for NAMPT and PPARγ) were defined according to BMI. GAPDH was used as a reference gene. The sample distribution, mean ratio values (horizontal lane) of each group and standard deviation (vertical lane) (SD) bars are shown in A-C. The correlation between ratio values and BMI scores are shown in D-F. p value <0.05 is indicated as *.

ADIPOQ, NAMPT (visfatin) and PPARγ expression were significantly lower in the synovium of the obese patients. The ratio of ADIPOQ/GAPDH, NAMPT/GAPDH and PPARγ/GAPDH negatively correlated with BMI score (Figure 4.1).

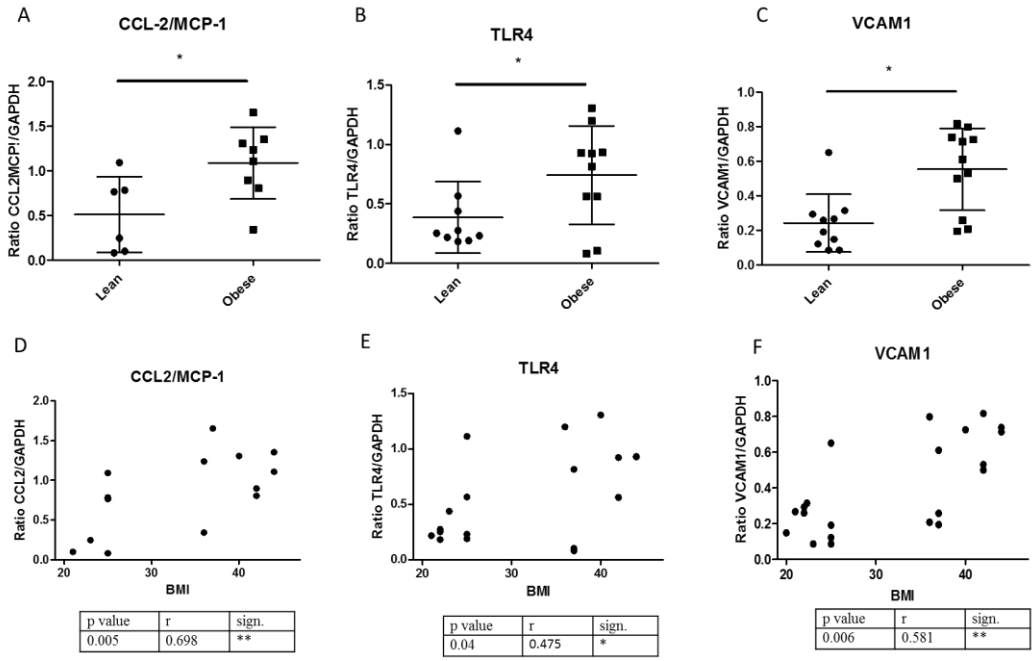



**Figure 4. 2 RT-PCR semi-quantitative analysis of CCL2/MCP-1, VCAM1 and TLR4 expression in synovium from OA patients.**
Two groups termed "Lean" (n=6 for CCL2/MCP-1, n=8 for TLR4 and n=10 for VCAM1 and "Obese" (n=8 for CCL2/MCP-1, n=10 for TLR4 and n=11 for VCAM1) were divided according to BMI. GAPDH was used as a reference gene. The sample distribution, mean ratio values (horizontal lane) of each group and standard deviation (vertical lane) (SD) bars are shown in A-C. The correlation between ratio values and BMI score were shown in D-F. p value <0.05 is indicated as *.

Chemokine CCL2/MCP-1 gene and two surface molecules: VCAM1 and TLR4 were significantly higher in the synovial tissue from the obese group compared to the lean one. The ratio of CCL2/MCP-1/GAPDH TLR4/GAPDH and VCAM11/GAPDH positively correlated with BMI score (Figure 4.2).

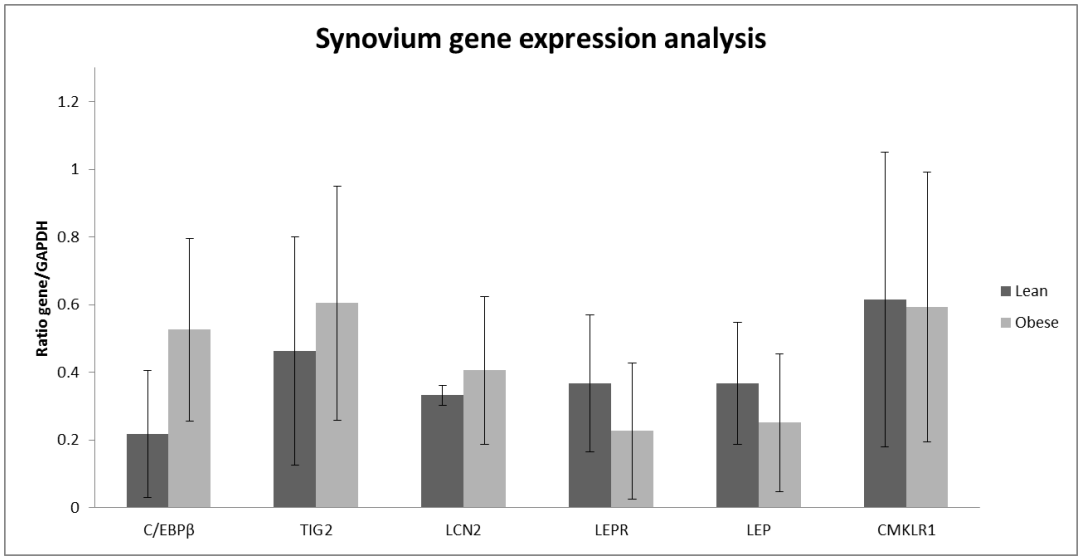



**Figure 4. 3 Semi-quantitative PCR analysis of genes in synovium obtained from OA patients.** Two groups termed "Lean" n=8 and "Obese" n=12 were defined according to BMI. GAPDH was used as a reference gene. The mean ratio values of each group and standard deviation (SD) bars are shown.

The other obesity-related genes investigated in the present study did not show any significant differences.

## 4.2.2 Quantitative Real Time qRT PCR analysis

The 6 candidate genes screened for by RT-PCR showing correlation with BMI were further analysed using quantitative Real Time PCR reaction. cDNA from a new set of 12 synovial samples for each group (lean and obese) were analysed in triplicate and gene expression was normalized to the housekeeping gene B2M (Figure 4.4).

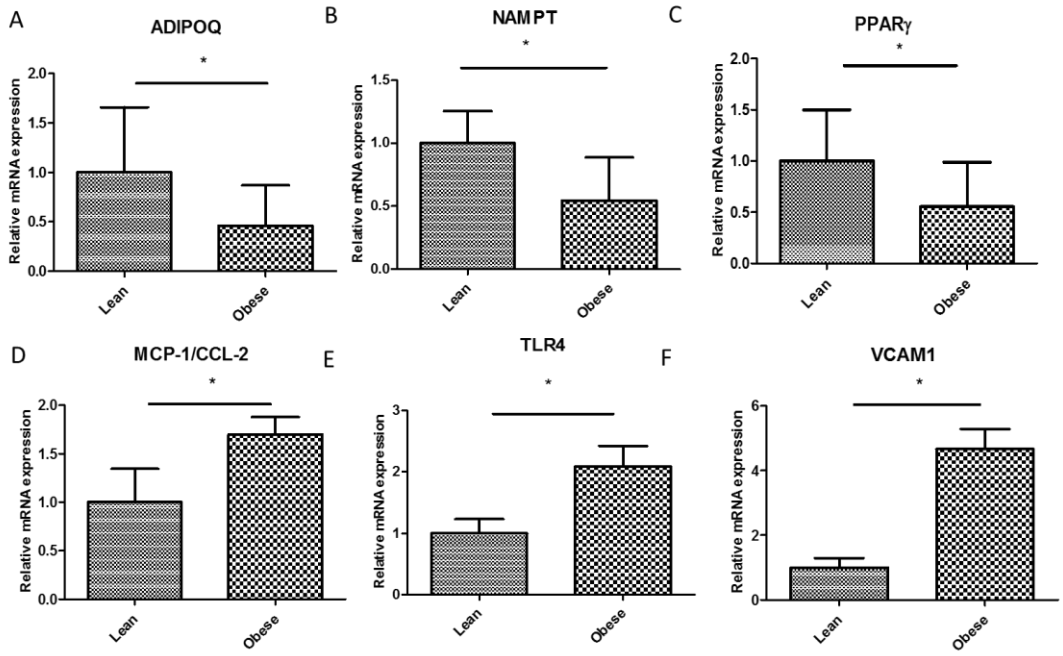



**Figure 4. 4 Quantitative PCR analysis of ADIPOQ, NAMPT, PPARγ, MCP1/CCL2, TLR4 and VCAM1 gene expression in synovium obtained from OA patients.**
Quantitative PCR analysis of ADIPOQ (A), NAMPT (B), PPARγ (C), MCP-1/CCL-2 (D), TLR4 (E), VCAM1 (F) expression in synovium obtained from different OA patients. Two groups termed "Lean" n=10 and "Obese" n=12 were defined according to BMI as before. B2M was used to normalise gene expression. The -ΔΔCT method was used. The mean value of Lean Group was used as a calibrator to investigate fold change of gene expression. The ratio values of each group and SEM bars are shown. p values < 0.05 were considered significant and indicated as *.

Real Time PCR results (Figure 4.4) confirmed the semi-quantitative RT-PCR data. ADIPOQ, NAMPT and PPARγ were expressed at a lower level while CCL2, TLR4 and VCAM1 were expressed at higher level in the synovium of Obese as compared to Lean OA patients.

## 4.3 Protein expression in synovium

### 4.3.1 Adiponectin protein expression in synovium

Immunohistochemistry of synovial histology sections showed adiponectin protein expression (Figure 4.5-4.6).

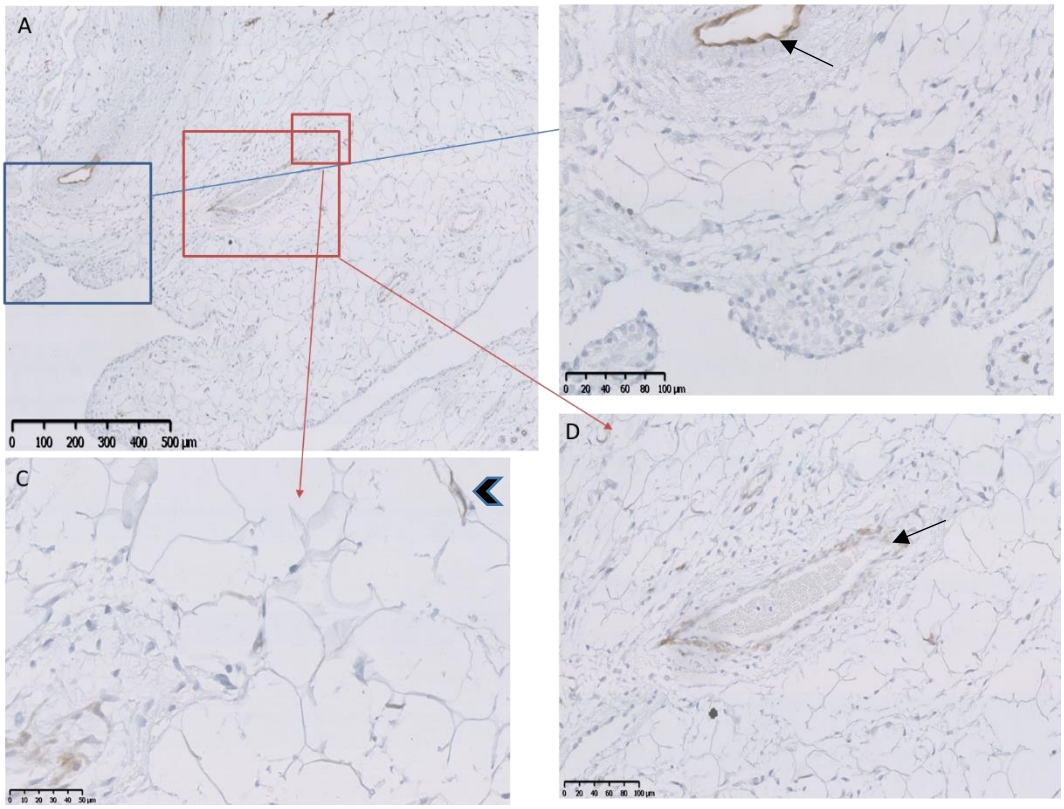



**Figure 4. 5 Adiponectin expression in synovium.**
IHC staining of paraffin-embedded synovium confirmed expression of adiponectin (brown colour). Different localisation within the tissue can be detected (A-D) perivascular (black arrow in B, D) and in adipocytes (black arrowhead C). (For non-staining and isotype controls see Appendix 7).

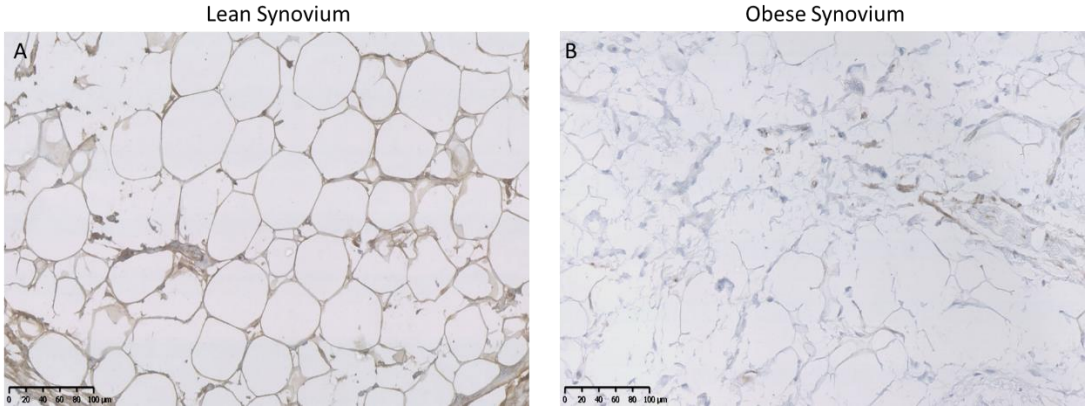



**Figure 4. 6 Adiponectin expression in synovium from Lean and Obese OA patients.**
Representative IHC adiponectin staining (brown colour/black arrows) for synovium from Lean (A) and Obese (B) OA patients.

Adiponectin is present in the perivascular and adipose tissue areas of synovium of all patients tested (Figure 4.5). Adiponectin can be detected in both lean and obese patients synovium (Figure 4.6). Western Blot analysis of synovial tissue protein lysates was conducted to show differences between lean and obese OA subjects (Figure 4.7).

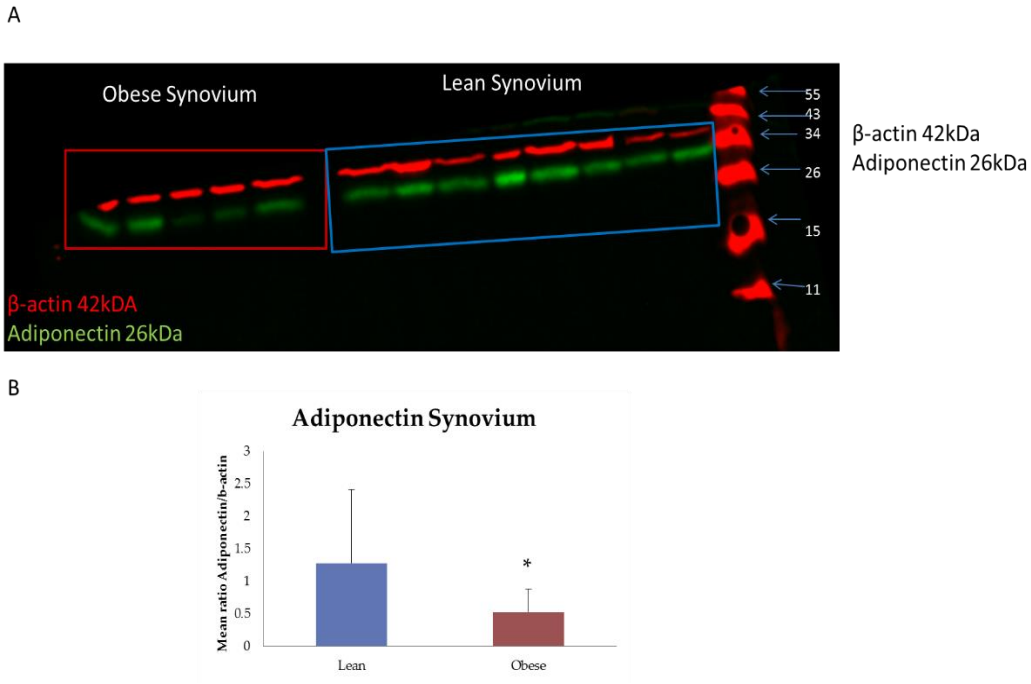



**Figure 4. 7 Semi-quantitative WB analysis of Total Adiponectin protein expression in synovial protein lysates from Lean and Obese OA patients.**
Representative WB result (A) and densitometry analysis (B) showing adiponectin expression in synovium from Lean and Obese OA patients. Densitometry data are presented as the mean value of the ratios of adiponectin to β-actin for each group ± SD (n=10 for each group). p values < 0.05 were considered significant and indicated *.

WB analysis showed there was a significant difference in total adiponectin expression between synovium of lean and obese patients. Synovium from obese subjects with OA expressed less adiponectin than from lean patients (Figure 4.7).

Adiponectin secretion was also confirmed by enzyme-linked immunoassay (ELISA) technique (Figure 4.8). Synovium explants were cultivated in serum free media for 3 days (ex vivo culture). The conditioned media were collected and adiponectin release was measured by ELISA.

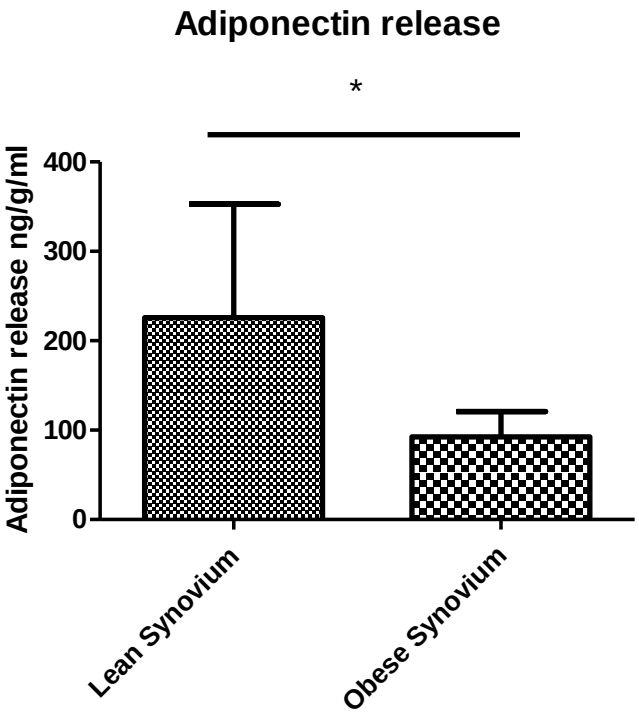



**Figure 4. 8 Ex vivo adiponectin release by synovium from Lean and Obese OA patients.** Quantification of adiponectin release into the media by synovium tissue explants after 3 days in serum free media measured by ELISA. Each tissue explant was weighted and cultivated in duplicate. ELISA detection was conducted in duplicates for each sample. The duplicate has been averaged and results are shown as mean values from the group ± SD (n=5 for each group).

ELISA confirmed that adiponectin was produced by synovium from lean at a significantly higher level than from obese OA patients (Figure 4.8).

### 4.3.2 PPARγ protein expression in synovium

The PPARγ protein expression was assessed in synovium in histology sections using an immunohistochemical technique (Figure 4.9-4.10).

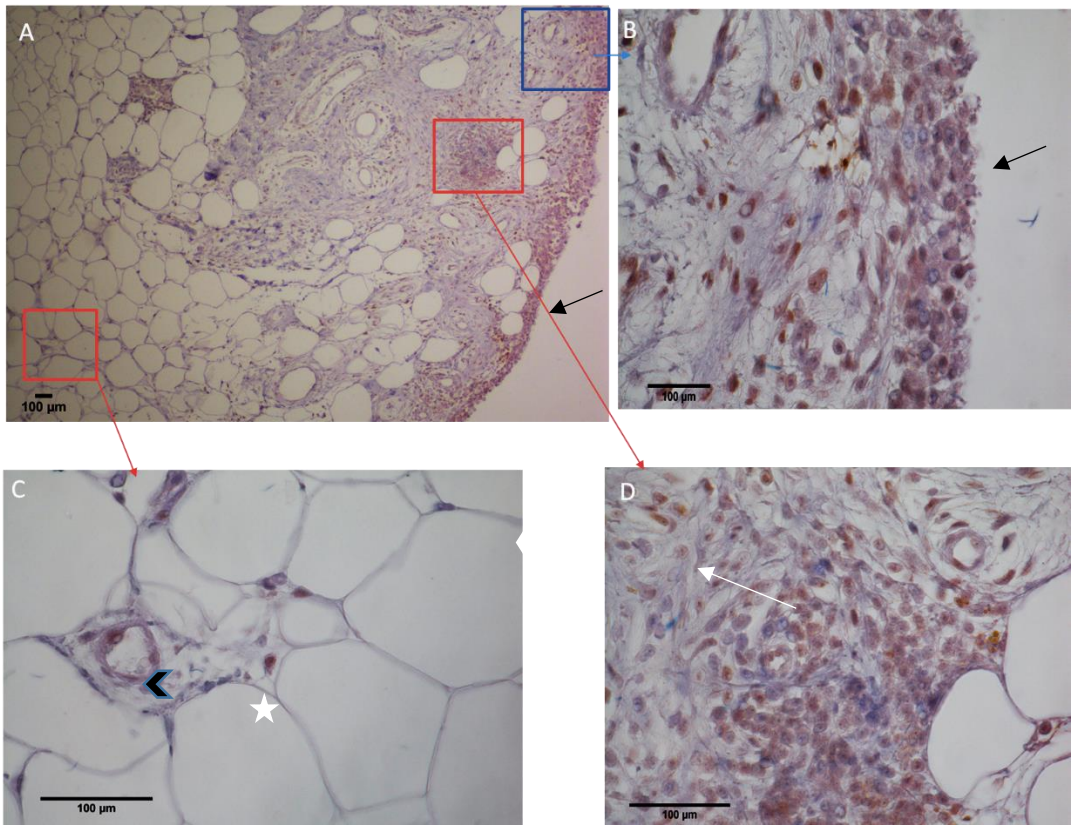



**Figure 4. 9 PPARγ expression in synovium.**
IHC staining of paraffin-embedded synovium confirmed expression of PPARγ (brown colour). Different localisation within the tissue could be detected (A-D). It was seen in the synovial membrane (black arrows in A,B) perivascular area (black arrowhead in C), in fatty (asterix in C) and fibrotic tissue (white arrow in D). (For non-staining and isotype controls see Appendix 7).

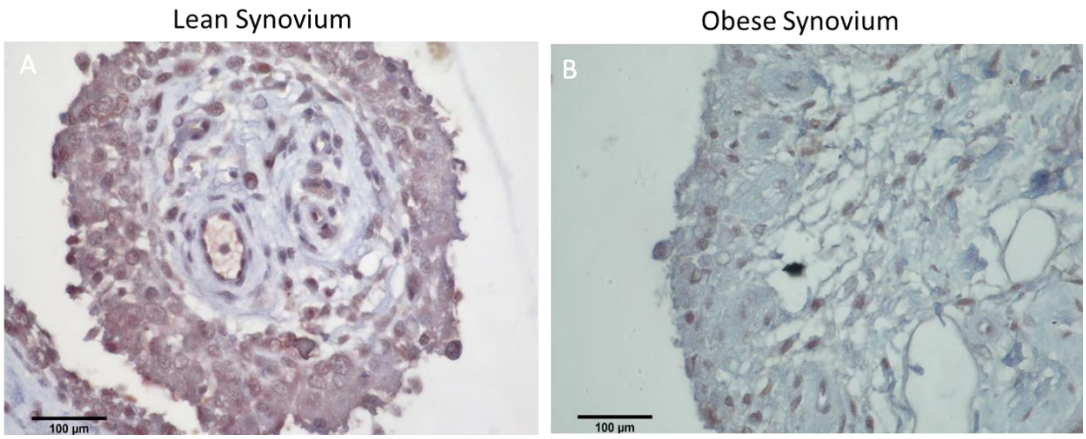



**Figure 4. 10 PPARγ expression in synovium from Lean and Obese OA patients.**
Representative IHC PPARγ staining (brown colour/black arrows) for synovium from Lean (A) and Obese (B) OA patients.

PPARγ was present in the synovial membrane, the perivascular area, adipose and fibrotic tissue (Figure 4.9). PPARγ is present in the synovium of both lean and in obese OA subjects (Figure 4.10). The differences between two groups were analysed by Western Blot technique (Figure 4.11).

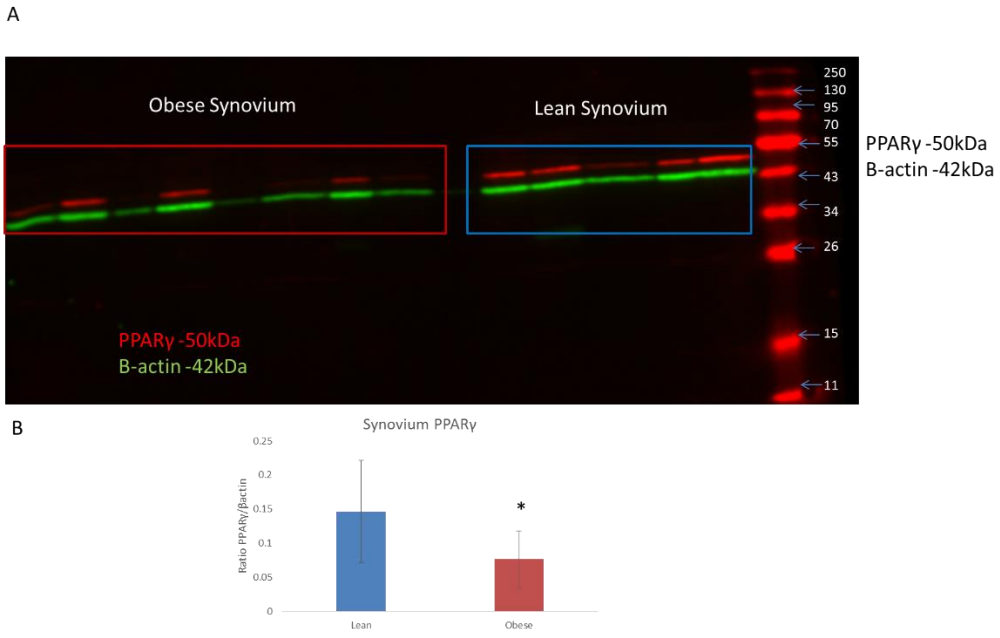



**Figure 4. 11 Semi-quantitative WB analysis of PPARγ expression in synovium from Lean and Obese OA patients.**
Representative WB result (A) and densitometry analysis (B) demonstrated significantly different PPARγ expression in synovium from Lean and Obese OA patients. Densitometry data is present as mean value of the ratios of PPARγ to β-actin for each group ± SD (n=10 for each group).
p values <0.05 were considered significant and indicated *.

Semi-quantitative WB results confirmed the PCR data that PPARγ was significantly lower in the synovium from Obese in comparison to Lean OA patients (Figure 4.11).

### 4.3.3 Visfatin protein expression in synovium

Immunohistochemical staining of synovial explants (Figures 4.12-4.13).

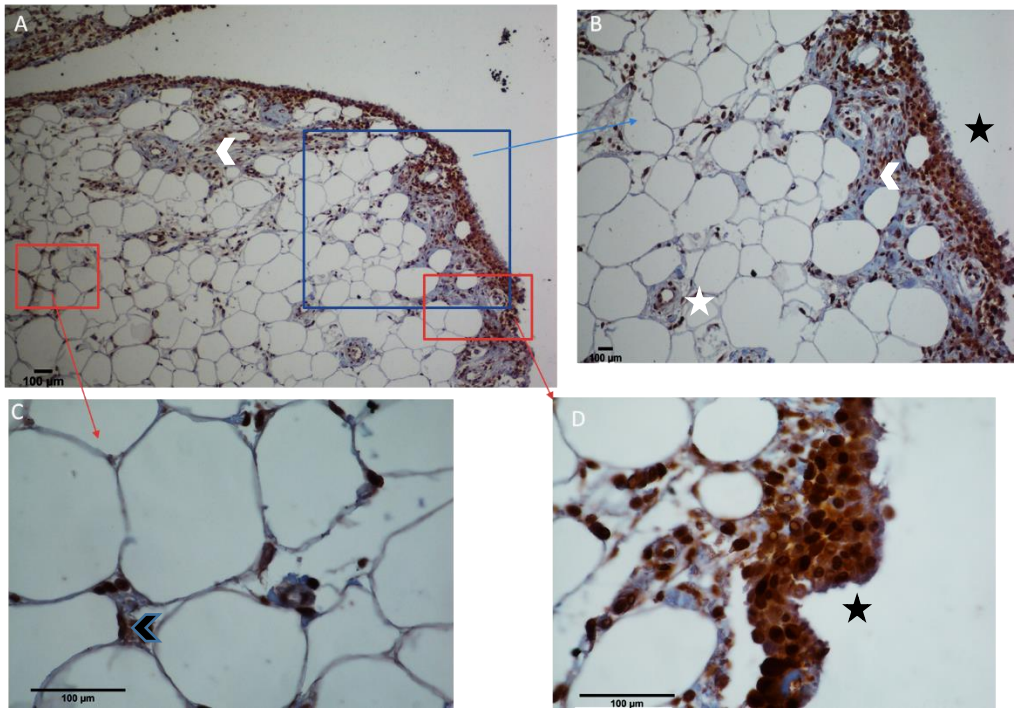



**Figure 4. 12 Visfatin expression in synovium.**
IHC staining of paraffin-embedded synovium confirmed the expression of visfatin (brown colour). Different localisation within the tissue was detected (A-D) synovial membrane (black asterix), perivascular area (white asterix) (B), in adipose (black arrowhead in C) and fibrotic tissue (white arrowhead in A,B). (For non-staining and isotype controls see Appendix 7).

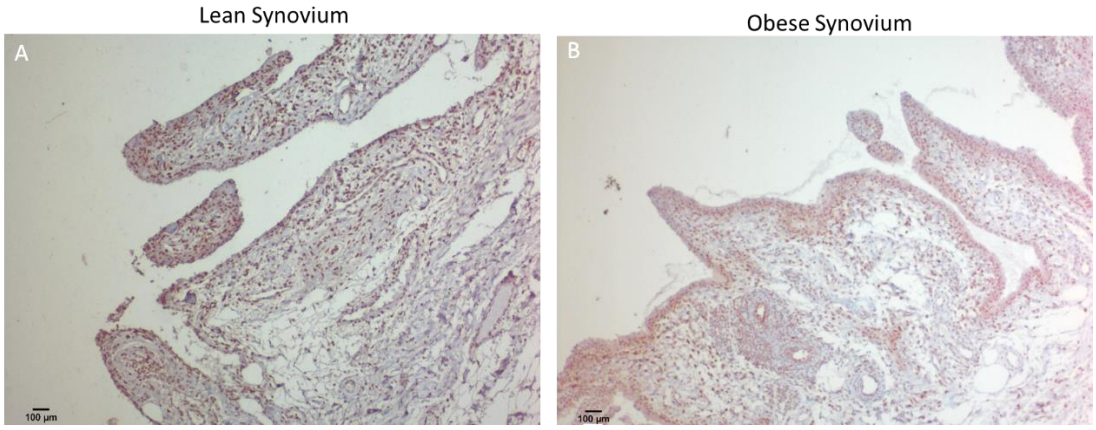



**Figure 4. 13 Visfatin expression in synovium from Lean and Obese OA patients.**
Representative IHC visfatin staining (brown colour/black arrows) for synovium from Lean (A-C) and Obese (D-F) OA patients.

IHC staining showed that visfatin was present in both the lining and sublining area of the synovial membrane, and in perivascular, fibrotic and adipose tissue areas (Figure 4.12).

Visfatin can be detected in the synovium of both lean and obese OA subjects (Figure 4.13).

Western Blot technique was used to analyse visfatin expression in synovial tissue lysates (Figure 4.14).

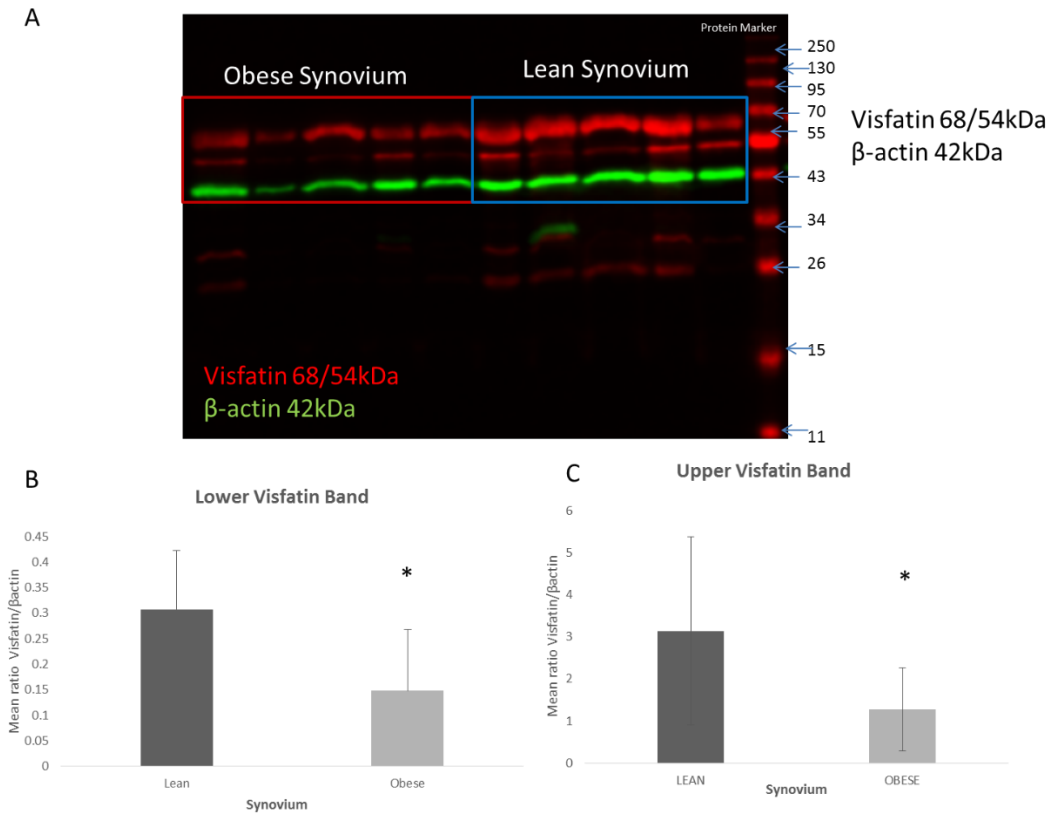



**Figure 4. 14 Semi-quantitative WB analysis of visfatin expression in synovium from Lean and Obese from OA patients.**
Representative WB result (A) and densitometry analysis (B, C) demonstrated significantly higher visfatin expression in Lean compared to Obese synovium obtained from OA patients. Densitometry data are present as mean values of the ratio of visfatin to β-actin for each group ± SD (n=10 for each group). p values < 0.05 were considered significant.

Unexpectedly two visfatin bands were detected in synovial protein tissue lysates. The higher molecular weight (MW) band (about 15-20kDa bigger) was much more highly expressed than the lower one. Semi-quantitative analysis showed that expression of both lower and upper bands was reduced in obese compared to lean OA patients confirming the data obtained in PCR analysis (see Figure 4.14).

WB analysis confirmed previous PCR data that visfatin had a significantly lower expression in the synovium of Obese OA patients in comparison to Lean ones.

### 4.3.4 CCL2 protein expression in synovium

CCL2 production by synovium was analyzed using the quantitative ELISA technique (Figure 4.15). Synovium explants were cultivated in serum free media for 3 days (72h ex vivo culture). Conditioned media were collected after that and CCL2 release was measured by ELISA.

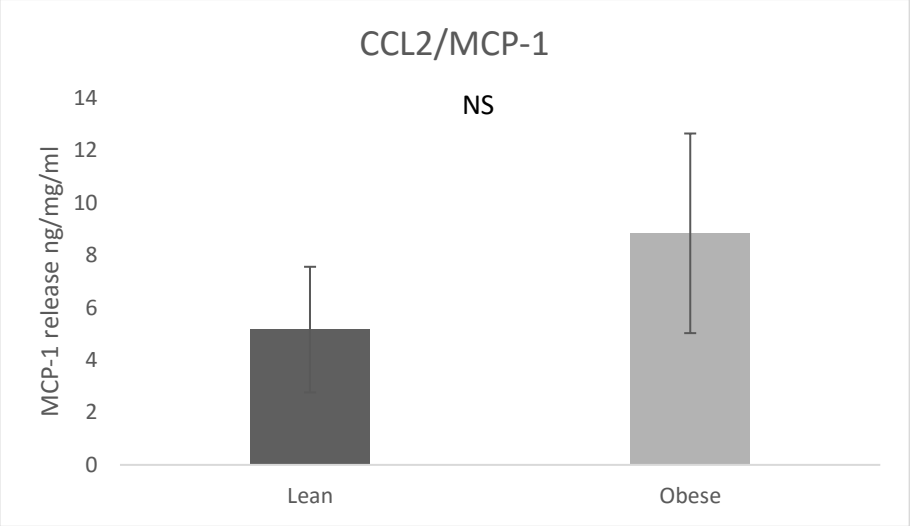



**Figure 4. 15 CCL-2/MCP-1 release to the media from cultured Synovial explants from Lean and Obese OA patients.**
Quantification of CCL-2 release into the media by synovium tissue explants after 3 days in serum free media measured by ELISA. Each tissue explant was weighted and cultivated in duplicates. ELISA detection was conducted in duplicates for each sample. Results are shown as mean values ± SD (n=5 for each group). NS – non-significant.

ELISA method showed that CCL-2/MCP-1 was produced by synovium (Figure 4.15). There was a trend towards higher CCL2 production by obese synovium but it was not statistically significant (p=0.08).

### 4.3.5 TLR4 protein expression in synovium

The expression of TLR4 protein was assessed in synovial histology sections using immunohistochemistry (Figure 4.16-4.17).

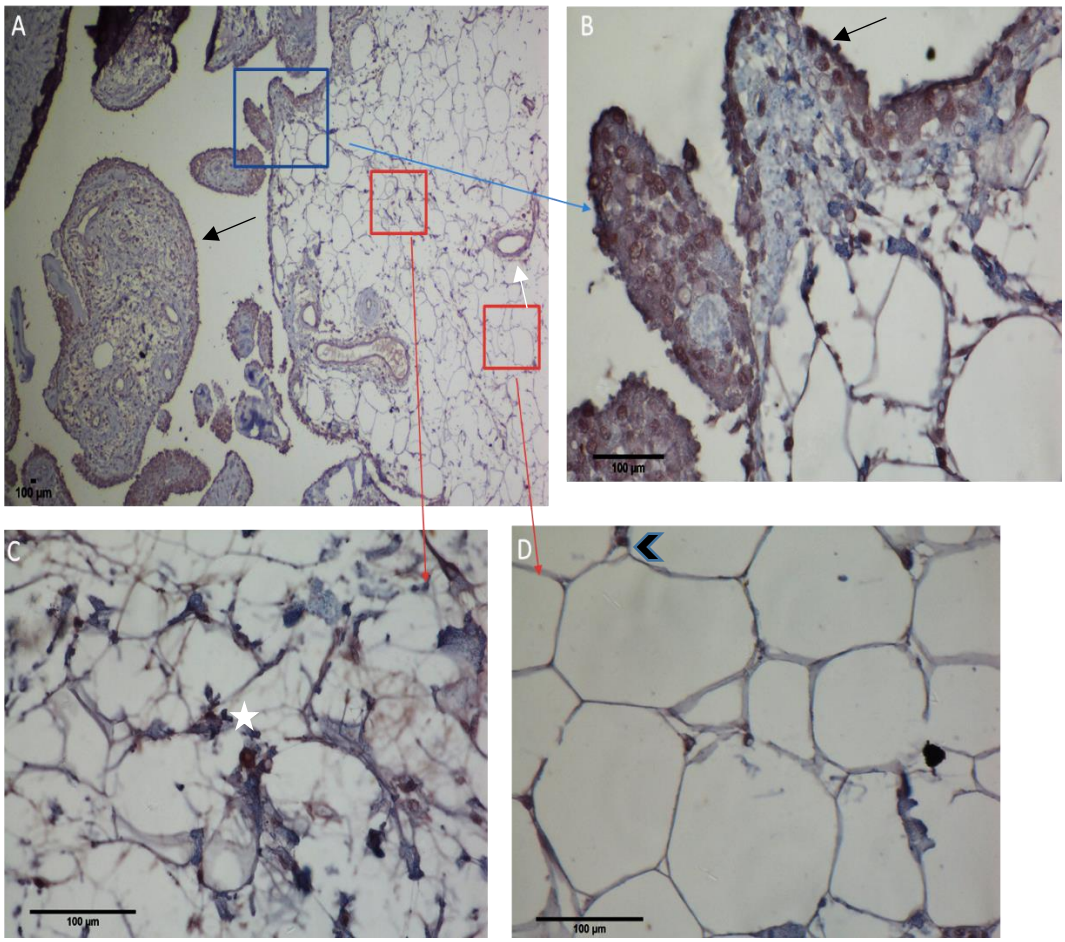



**Figure 4. 16 TLR4 expression in synovium.**
IHC staining of paraffin-embedded synovium confirmed expression of TLR4 (brown colour). Different localisation within the tissue was observed (A-D): synovial membrane (black arrows in A,B), perivascular area (white arrow in A), fibrotic tissue (asterix in C) and in adipocytes (black arrowhead in D). (For non-staining and isotype controls see Appendix 7).

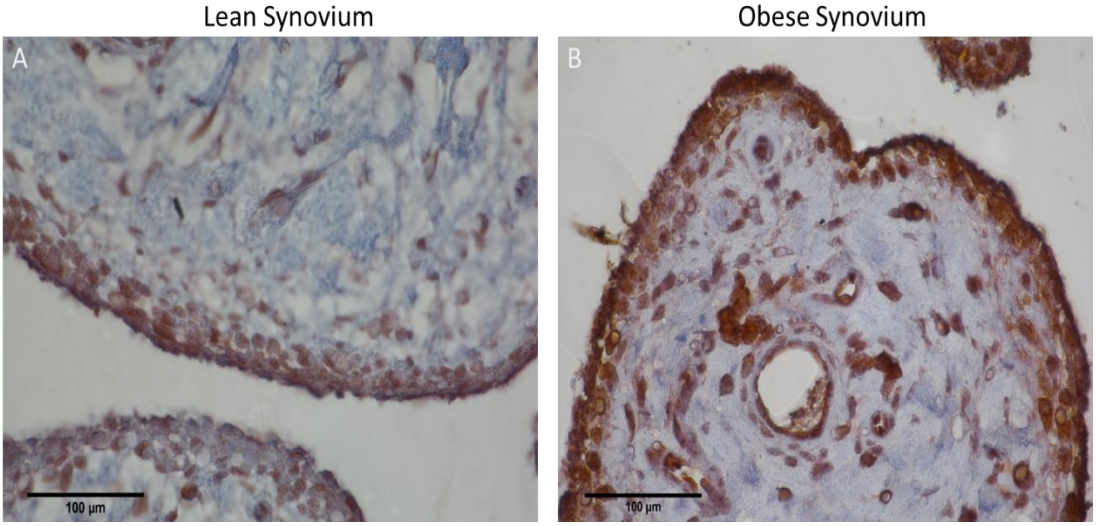



**Figure 4. 17 TLR4 expression in synovium from Lean and Obese OA patients.**
Representative IHC TLR4 staining (brown colour) for Lean (A-C) and Obese (D-F) synovium.

TLR4 was present in the synovial membrane, perivascular area, adipose and fibrotic tissue (Figure 4.16). TLR4 is expressed in synovium from both lean and obese OA subjects (Figure 4.17).

The TLR4 expression in the stromovascular fraction (SVF) of synovium was investigated by Flow Cytometry (Figure 4.18). The gating strategy is shown in Appendix 5. The SVF fraction was analysed by Forward Scatter (FCS) and Side Scatter (SCC). Cell debris was excluded. Single marker staining was visualised with Phycoerythrine (PE) fluorochrome antibody against TLR4 together with an isotype antibody control (IgG-PE) (negative control). The percentage of $TLR4^{+}$ cells per total number of SVF cells was calculated for each patient (as shown in Figure 4.18). The Mean Fluorescent Intensity (MFI) of TLR4 expression in analysed samples was also investigated.

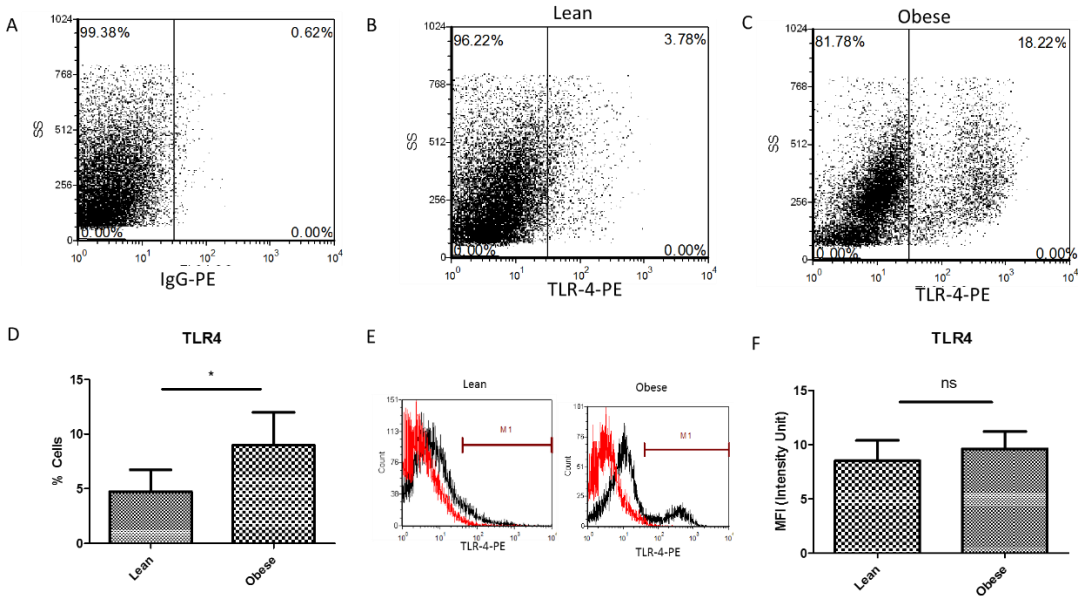



**Figure 4. 18 Flow Cytometry analysis of TLR4 protein expression in SVF fraction of synovium from Lean and Obese OA patients.**
The isotype control staining shown in A. The representative dot plot analysis for Lean (B) and Obese (C) patients synovium. The percentage of $TLR4^{+}$ cells per total number of SVF cells was calculated for each patient. The mean percentage values of each group (n=7 for each group) and standard deviation (SD) bars are shown in D. Example of Mean Fluorescent Intensity analysis for TLR4 expression shown in E. The mean MFI value for each group ± SD (n=7 for each group) shown in F. p values < 0.05 were considered significant and indicated *, ns- non-significant.

The data shows that a significantly higher frequency of cells expressing TLR4 in obese as compared to lean OA patients. Furthermore, analysis of Mean Fluorescence Intensity (MFI) (a measure of the intensity of expression) suggests that there is no significant difference in intensity of TLR4 expression in cells from obese OA patients synovium (Figure 4.18).

### 4.3.6 VCAM-1 protein expression in synovium

The VCAM-1 protein expression was assessed in synovial histology sections using an immunohistochemical technique (Figure 4.19-4.20).

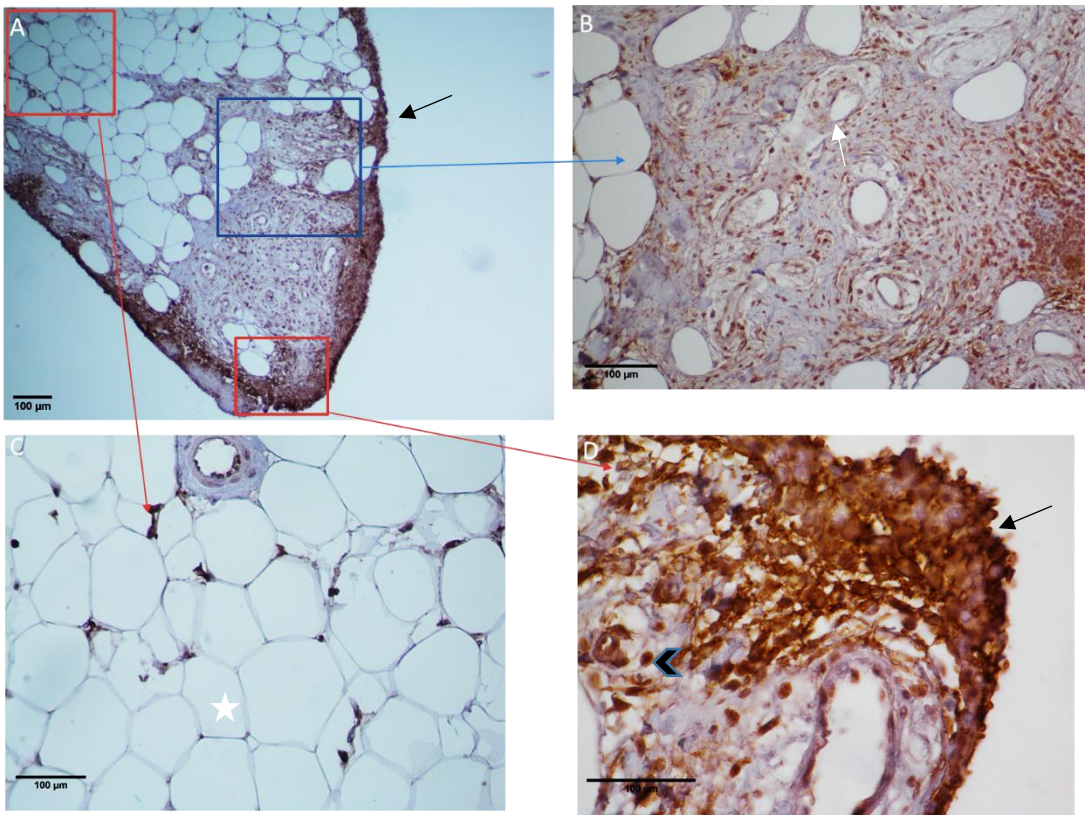



**Figure 4. 19 VCAM-1 expression in synovium.**
IHC staining of paraffin-embedded synovium confirmed expression of VCAM-1 (brown colour). Different localisation within the tissue was observed (A-D). It was detected in the synovial membrane (black arrows in A,D) and perivascular area (white arrow in B), in adipocytes (asterix in C) and in the fibrotic tissue (black arrowhead in D). (For non-staining and isotype controls see Appendix 7).

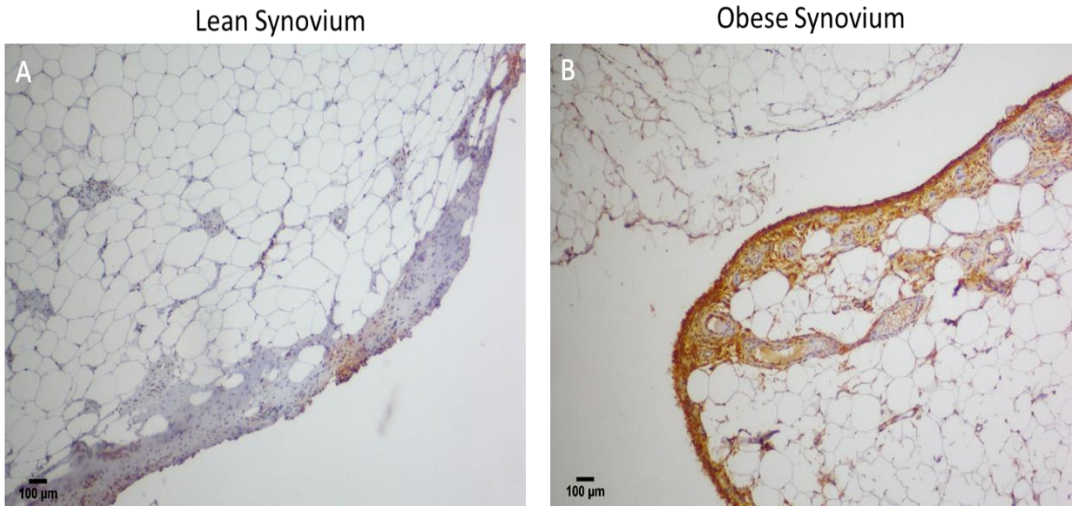



**Figure 4. 20 VCAM-1 expression in synovium from Lean and Obese OA patients.**
Representative IHC VCAM-1 staining (brown colour/black arrows) for Lean (A-C) and Obese (D-F) synovium.

VCAM-1 was present in the synovial membrane, perivascular area and in the adipose and fibrotic tissue (Figure 4.19). VCAM-1 can be detected in the synovium of both lean and obese OA subjects (Figure 4.20).

VCAM-1 expression in SVF of synovium was investigated by Flow Cytometry (Figure 4.21). The gating strategy was as shown in Appendix 5. Single marker staining was visualised using Phycoerythrine (PE) fluorochrome antibody against VCAM-1 with an isotype control (IgG-PE). The percentage of VCAM-$1^{+}$ cells per total number of SVF was calculated for each patient. The Mean Fluorescent Intensity (MFI) of VCAM-1 expression in analysed samples was also investigated.

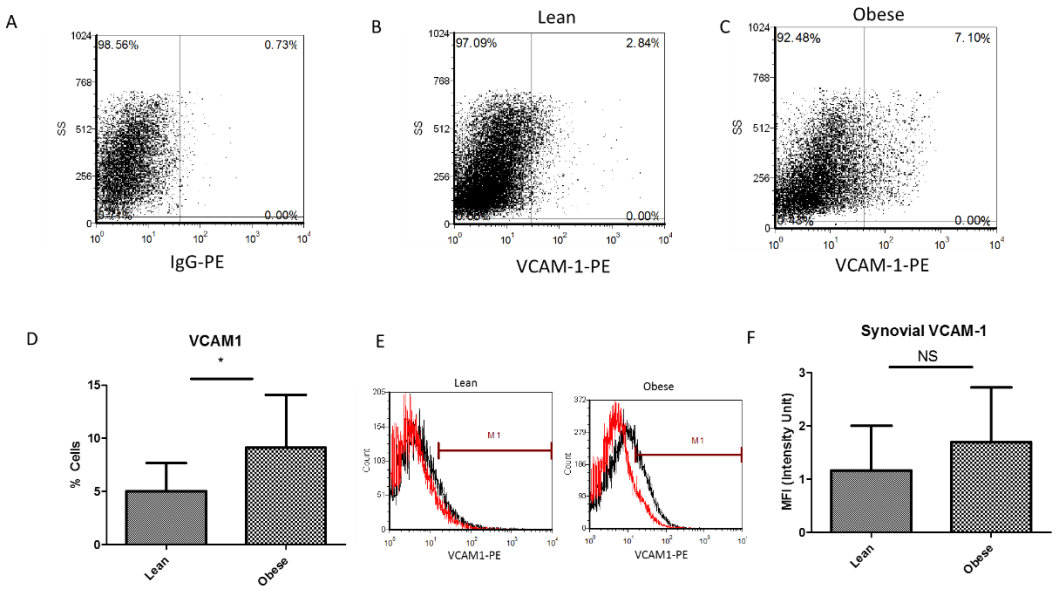



**Figure 4. 21 Flow Cytometry analysis of VCAM-1 protein expression in SVF fraction of synovium of Lean and Obese OA patients.**
The isotype control staining is shown in A. The example of dot plots for Lean and Obese patients synovium shown in B,C. The percentage of VCAM-1+ cells per total number of SVF was calculated for each patient. The mean percentage values of each group (n=7 for each group) and standard deviation (SD) bars are shown in D. Example of Mean Fluorescent Intensity analysis for VCAM-1 expression shown in E. The mean MFI value for each group ± SD (n=7 for each group) shown in F. p values < 0.05 were considered significant and indicated *.

Data analysis showed that there is a significantly higher frequency of cells expressing VCAM-1 in obese as compared to lean OA patients. MFI analysis suggests that there is no significant difference in VCAM-1 intensity of expression (Figure 4.21).

## 4.4 Conclusion and Summary

In conclusion, obtained data revealed a number of important points:

1) The osteoarthritic synovial tissue obtained after TKR express obesity relevant markers
2) Synovium from Obese OA patients expressed pro-inflammatory markers including CCL2, VCAM-1, TLR4 at a higher level, both at mRNA and protein level than from Lean OA patients.
3) Synovium from Obese OA patients expresses particular adipose tissue related markers such as adiponectin, PPARγ and visfatin at a lower level than synovium from Lean patients both at mRNA and protein level.
4) Synovium from Obese Patients with OA released significantly lower levels of adiponectin than the synovium from Lean OA patients.

All of the above data could help to explain the correlation between obesity and OA. The genes and proteins investigated in this study suggest different molecular pathways in the synovium from obese and lean individuals. In particular, differences in adiponectin, PPARγ, visfatin, CCL-2, TLR4 and VCAM-1 expression in synovium were found to be altered and need to be investigated further.

# Chapter 5: The differences in obesity-related markers expression in Infrapatellar Fat Pad (IPFP) from lean and obese OA patients

## 5.1 Introduction

The Infrapatellar Fat Pad (IPFP) is considered to be an important secretory tissue within the knee joint. It produces such proteins as Prostaglandin 2 ($PGE_{2)}$, TNFα and $CCL_2$ and is postulated to play a significant role in the pathogenesis of OA (Clockaerts et al., 2012).

In the present study candidate genes and proteins, which could play an important role in the contribution of obesity to OA were screened. In samples from the Infrapatellar Fat Pad, the gene expression of the main adipokines(as in the previous chapters) ADIPOQ, NAMPT, TIG2, LCN2 and some of their reported receptors including ADIPOR1, ADIPOR2, LEPR and CMKLR1 were investigated. Additionally, gene expression of two important nuclear factors PPARγ and C/EBPβ were analysed. Furthermore, the important chemokine CCL2 and significant surface markers such as VCAM1 and TLR4 genes expression were also assessed.

At the protein level, three main candidates were investigated: adiponectin, visfatin and PPARγ.

### 5.1.1 Aims

The main goal of the presented study was to compare differences in obesity markers in the IPFP of patients undergoing knee replacement surgery.

1. To screen obesity-related candidate genes expressed by IPFP to determine if they have a potential role in the pathogenesis of OA.
2. To determine the relative expression of obesity-related biomarkers in the IPFP from obese and lean individuals with OA.

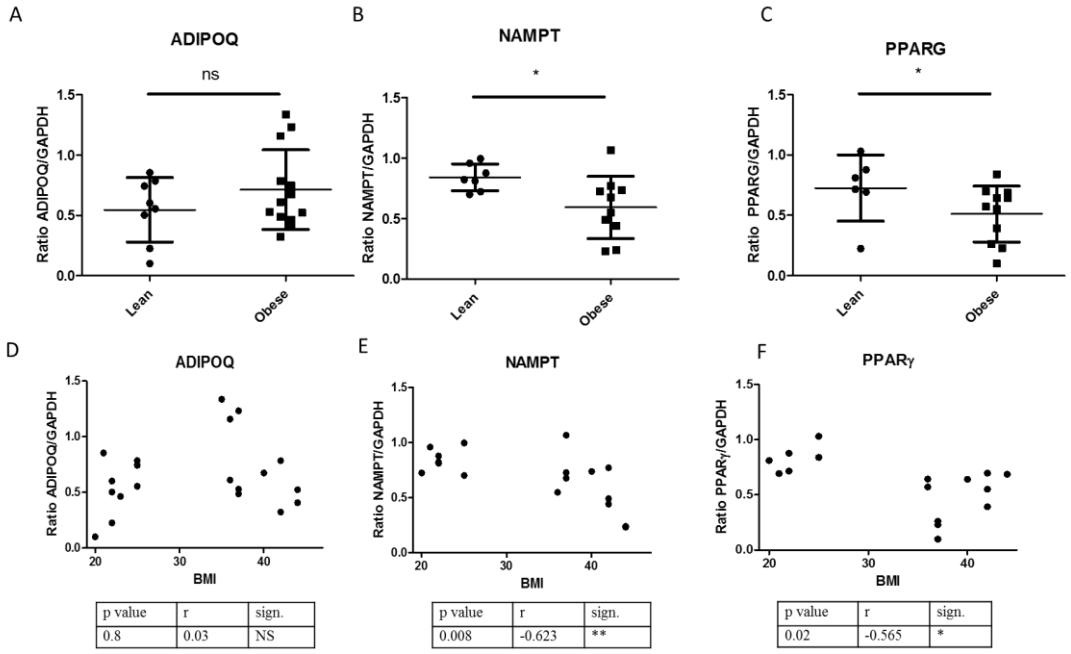
3. To investigate the differences between the two adipose tissue depots within the knee - synovium versus IPFP.

## 5.2 Infrapatellar Fat Pad gene expression analysis

IPFP biopsies were harvested from the knee joints of patients undergoing TKR (Total Knee Replacement) surgery. Patients were divided and histological analysis was conducted as previously described (representative histology is included in Appendix 8).

### 5.2.1 Semi-quantitative RT-PCR analysis

A semi-quantitative RT-PCR technique was used to screen candidate obesity marker to determine if there was a difference in gene expression in the IPFP from lean and obese patients (Figures 5.1-5.2)




| p value | r | sign. |
|---|---|---|
| 0.8 | 0.03 | NS |

| p value | r | sign. |
|---|---|---|
| 0.008 | -0.623 | ** |

| p value | r | sign. |
|---|---|---|
| 0.02 | -0.565 | * |

**Figure 5. 1 Semi-quantitative PCR analysis of ADIPOQ, NAMPT (visfatin) and PPARG genes in the IPFP from Lean and Obese patients with OA.**
Levels of adipokine genes in the infrapatellar fat pads obtained from two groups of OA patients; (A, C). "Lean" (n=9 for ADIPOQ n=8 for NAMPT and n=6 for PPARγ) and "Obese"(n=11 for ADIPOQ, n=10 for NAMPT and PPARγ). GAPDH was used as a reference gene. The sample distribution, mean ratio values (horizontal lane) for each group and standard deviation (SD) bars (vertical lane) are shown in A-C. The correlation between ratio values and BMI score are shown in D-F. p values < 0.05 were considered significant and indicated *. ns- non-significant.

The infrapatellar fat pad from all patients tested expressed ADIPOQ, NAMPT (visfatin) and PPARG genes. There was no significant difference in expression of

ADIPOQ between the obese and lean patient groups. The gene expression of NAMPT and PPARγ was significantly lower in IPFP from Obese OA patients in comparison to Lean ones (Figure 5.1).

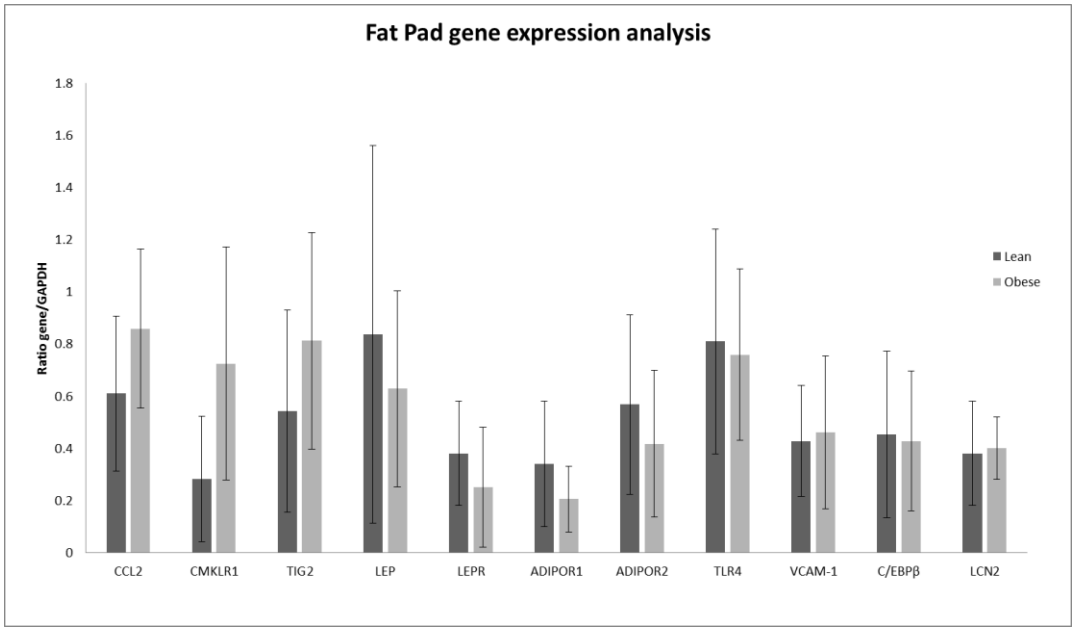



**Figure 5. 2 Semi-quantitative PCR analysis of genes expression in IPFP obtained from Lean and Obese subjects with OA.**
Possible candidate gene expression was analysed in the IPFP from OA patients. Two groups "Lean" n=8 and "Obese" n=12 were defined according to BMI. GAPDH was used as a reference gene. The mean ratio values of each group and standard deviation (SD) bars are shown.

Other candidate genes did not show significant differences (Figure 5.2).

## 5.2.2 Quantitative Real Time qRT PCR analysis

Three potential candidates for involvement in the interaction between OA and obesity were screened using the Real Time PCR reaction (Figure 5.3).

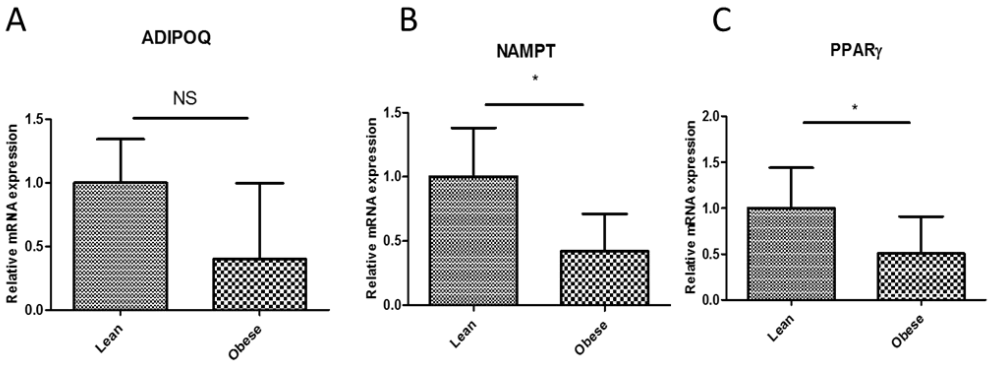



**Figure 5. 3 Real-Time PCR analysis of ADIPOQ, NAMPT and PPARγ expression in Infrapatellar Fat Pad from Lean and Obese OA patients.**
Quantitative analysis of ADIPOQ (A), NAMPT (B) and PPARγ (C) gene expression in IPFP from different OA groups: "Lean" n=10 and "Obese" n=10. B2M was used to normalise gene expression. The -ΔΔCT method was used. The mean value of Lean Group was used as a calibrator to investigate fold change of gene expression. The ratio values of each group and SEM bars are shown. p values < 0.05 were considered significant and indicated as * ns- non-significant.

Real-time PCR data confirmed the RT-PCR data. There was no significant difference between obese and lean groups in the expression of ADIPOQ gene in the IPFP, while significantly lower expression of PPARγ and NAMPT genes was detected in IPFP of Obese OA patients in comparison to Lean ones (Figure 5.3).

## 5.3 IPFP protein expression analysis

### 5.3.1 Adiponectin protein expression in IPFP

IPFP adiponectin protein expression was assessed in histology sections using immunohistochemistry (Figures 5.4-5.5).

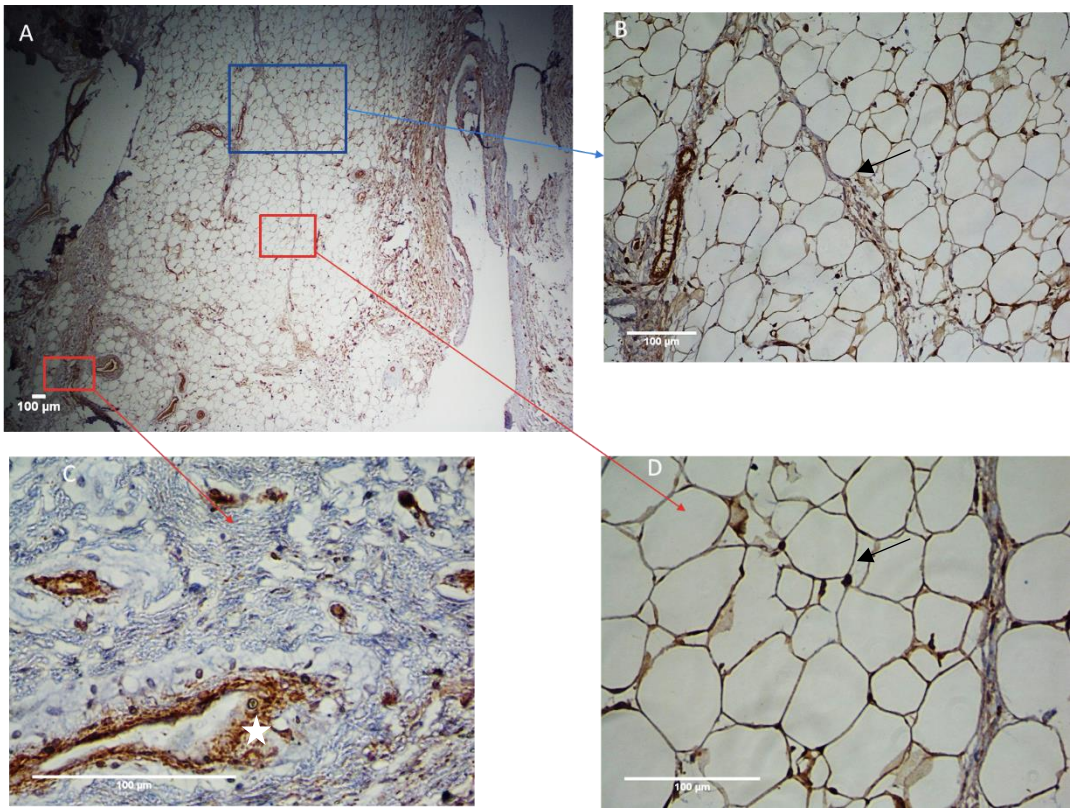



**Figure 5. 4 Adiponectin expression in IPFP.**
IHC staining of paraffin-embedded IPFP confirmed expression of adiponectin (brown colour). Different localisation of adiponectin within the tissue was observed (A-D): perivascular (asterix in C), within adipocytes (black arrows in B,D). (For non-staining and isotype controls see Appendix 7).

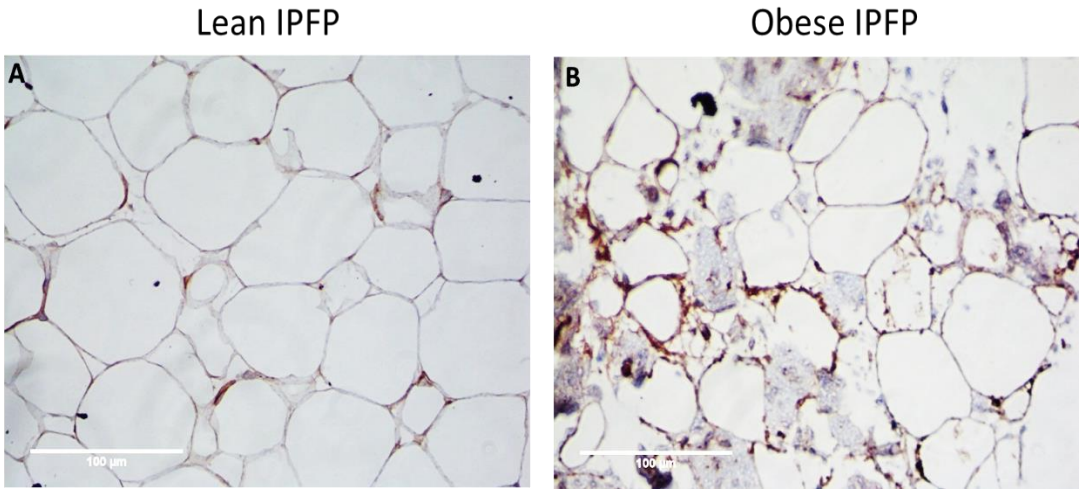


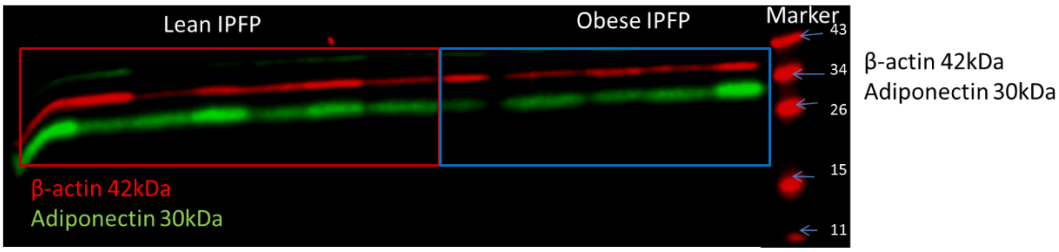

**Figure 5.5 Adiponectin expression in IPFP from Lean and Obese OA patients.**
Representative IHC adiponectin staining (brown colour) for IPFP from Lean (A-C) and Obese (D-F) patients.

IHC staining demonstrated ubiquitous adiponectin expression in IPFP tissue sections, within the vessels, but mainly in the adipocytes (Figure 5.4).

Adiponectin can be detected in IPFP of both lean and obese OA subjects (Figure 5.5).

Western Blot technique was used to analyse the difference in adiponectin expression between two groups (Figure 5.6).

A




B

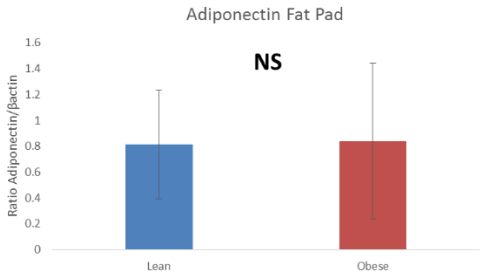



**Figure 5. 6 Semi-quantitative western blot analysis of total adiponectin expression in the IPFP from Lean and Obese OA patients.**
Representative western blot results (A) and densitometry analysis (B) of adiponectin expression in Infrapatellar Fat Pad (IPFP) from Lean and Obese OA patients. Densitometry data are presented as the mean value of the ratios of adiponectin to β-actin for each group ± SD (n=10 for each group). p values < 0.05 were considered significant.

WB analysis revealed that there is a significant difference in adiponectin expression in IPFP of lean and obese OA subjects.

Adiponectin secretion was confirmed by enzyme-linked immunoassay (ELISA) technique (Figure 5.7). IPFP explants were cultivated in serum free media for 3 days (ex vivo culture). Media was collected and adiponectin release was measured by ELISA.

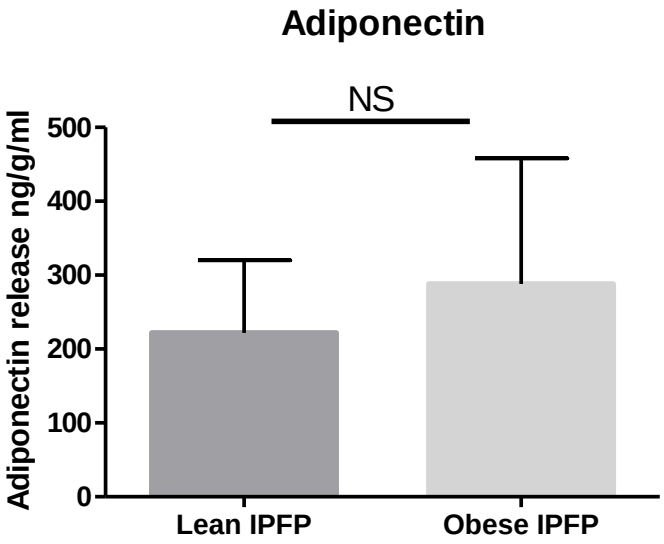



**Figure 5. 7 Ex vivo adiponectin release by IPFP from Lean and Obese OA patients.**
Quantification of the adiponectin release into media by IPFP tissue explants after 3 days in serum free media measured by ELISA. Each tissue explant was weighed and cultivated in duplicates. ELISA detection was conducted in duplicates for each sample. Results are shown as the mean values ± SD (n=5 for each group). NS- non-significant.

ELISA confirmed that adiponectin was produced by IPFP at similar levels in Lean and Obese patients with OA (Figure 5.7).

### 5.3.2 PPARγ protein expression in IPFP

The PPARγ protein expression was assessed in the IPFP in histology sections using an immunohistochemical technique (Figures 5.8-5.9).

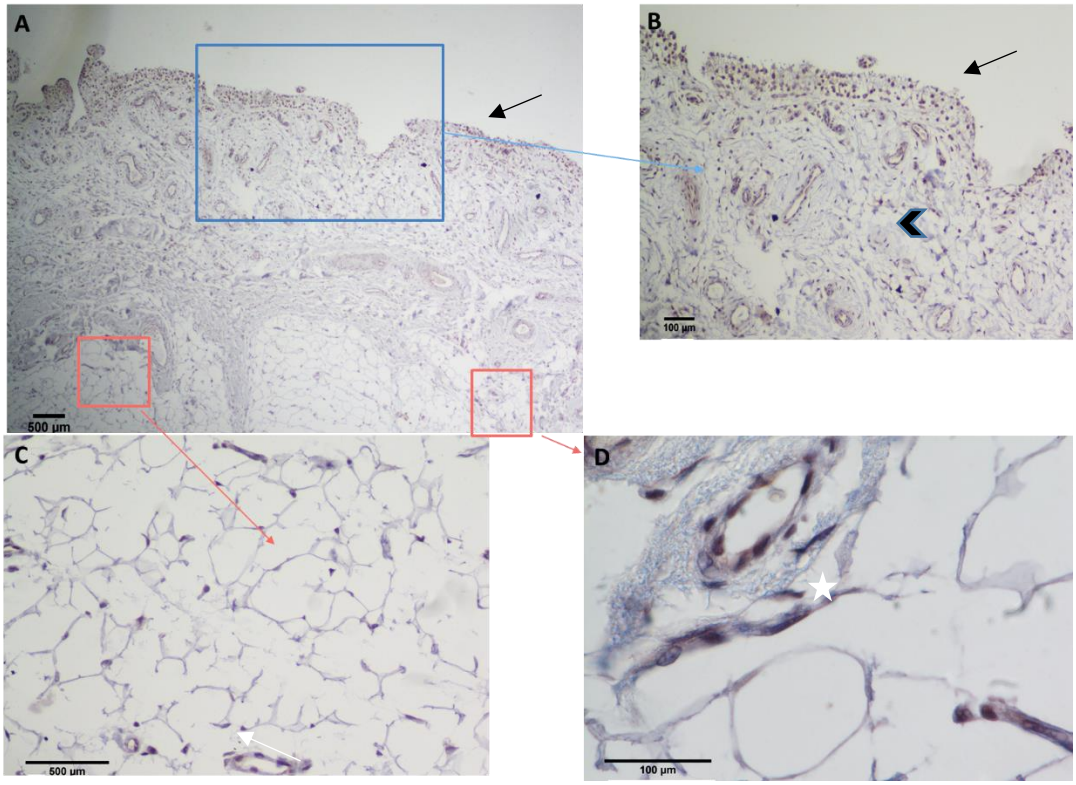



**Figure 5. 8 PPARγ expression in IPFP.**
IHC staining of paraffin-embedded IPFP confirmed expression of PPARγ (brown colour). Different localisation within the tissue was observed (A-D); synovial membrane (black arrow in A,B), in adipocytes (white arrow in C) perivascular (asterix in D) and fibrotic tissue (black arrowhead in D). (For non-staining and isotype controls see Appendix 8).

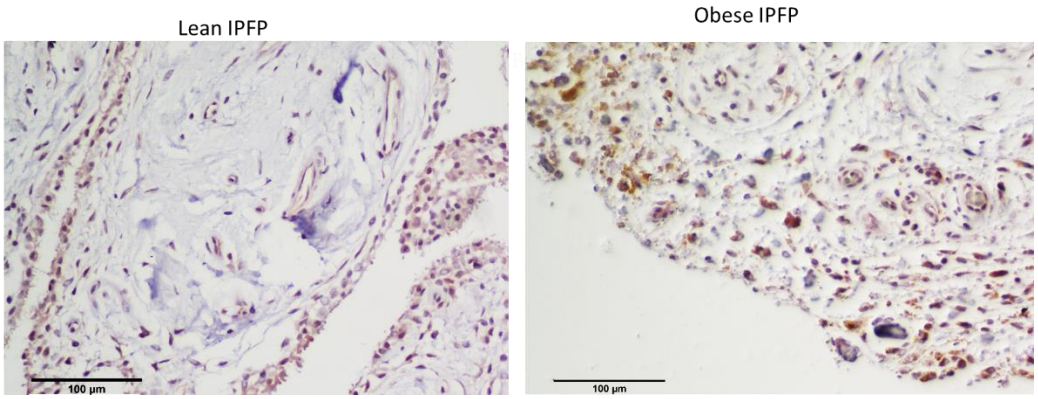



**Figure 5. 9 PPARγ expression in IPFP from Lean and Obese patients.**
Representative IHC PPARγ staining (brown colour/black arrow) for IPFP from Lean (A-C) and Obese (D-F) patients.

IHC staining of paraffin-embedded IPFP confirmed expression of PPARγ. Different localisation was observed in the synovial membrane, adipocytes, perivascular and fibrotic tissue (Figure 5.8). PPARγ is expressed in IPFP of both lean and obese OA subjects (Figure 5.9).

To explore the hypothesis that PPARγ expression was lower in infrapatellar fat pads of obese patients, semi-quantitative WB analysis was conducted (Figure 5.10).

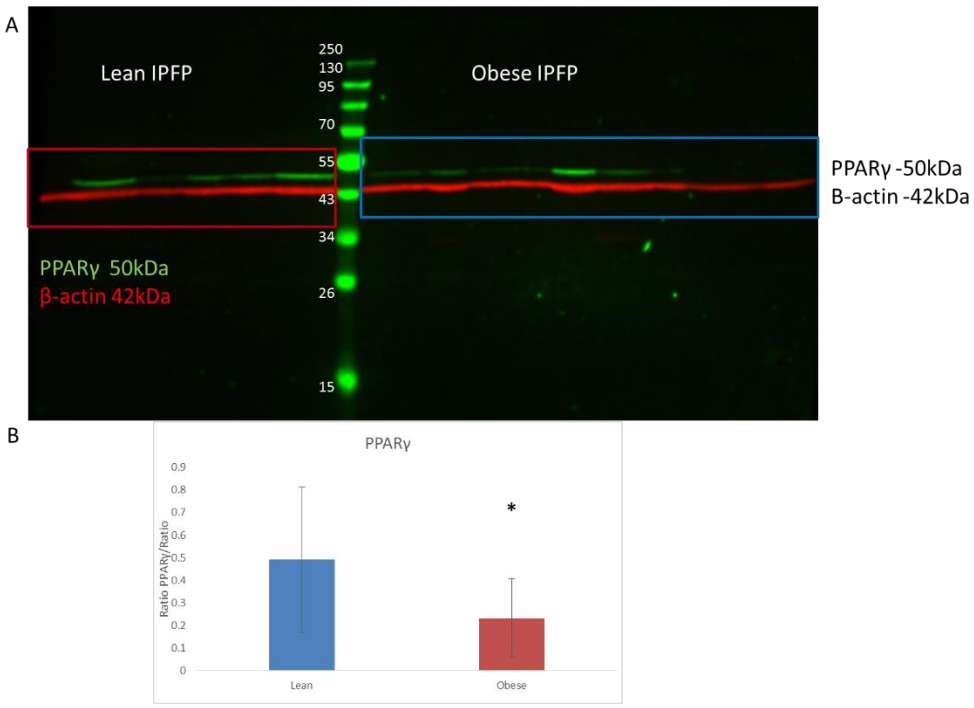



**Figure 5. 10 Semi-quantitative Western Blot analysis of PPARγ expression in IPFP from Lean and Obese OA patients.**
Representative WB result (A) and densitometry analysis (B) PPARγ expression in IPFP from Lean and Obese OA patients. Densitometry data are presented as the mean value of the ratio of PPARγ to β-actin for each group ± SD (n=10 for each group).

Semi-quantitative WB results confirmed the PCR data that PPARγ was significantly lower in the IPFP from Obese in comparison to Lean OA patients (Figure 5.13).

### 5.3.3 Visfatin protein expression in IPFP

The visfatin protein expression was assessed in IPFP in histology sections using immunohistochemistry (Figure 5.11-5.12).

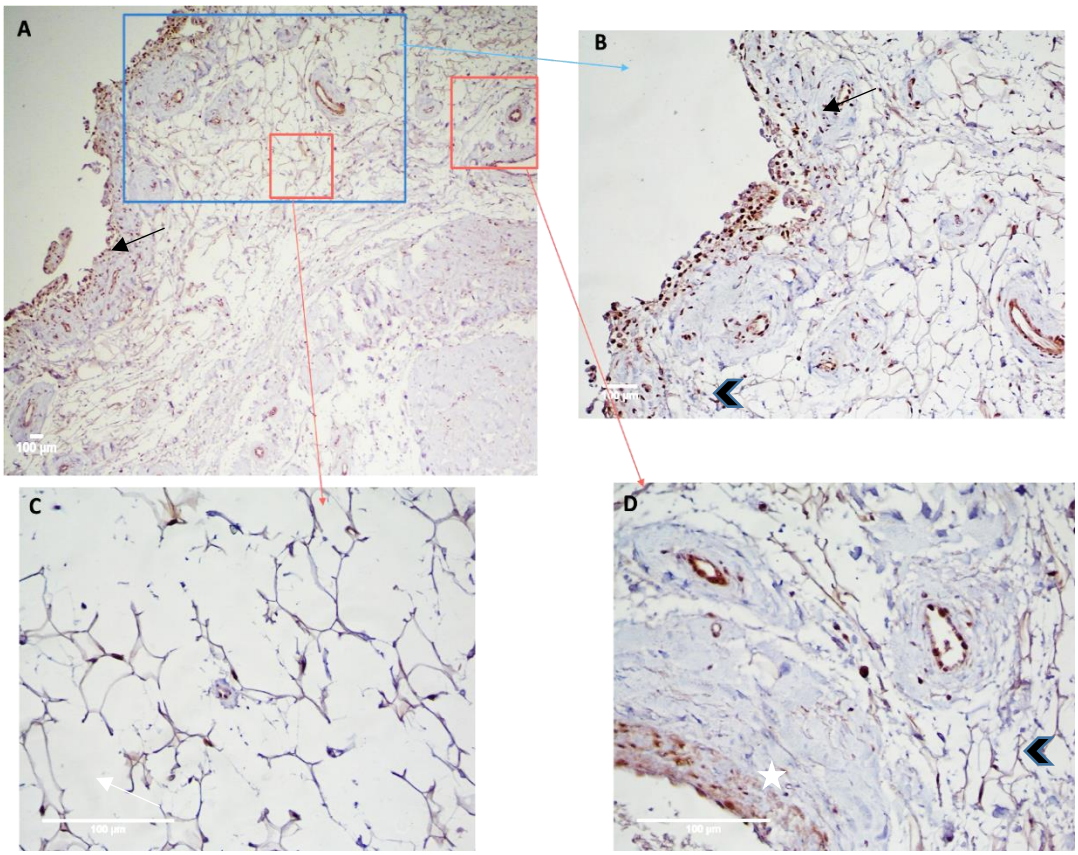



**Figure 5. 11 Visfatin expression in IPFP.**
IHC staining of paraffin-embedded IPFP confirmed expression of visfatin (brown colour). Different localisation within the tissue was observed (A-D); synovial membrane (black arrows in A,B), adipocytes (white arrow in C) perivascular (white asterix in D) and fibrotic tissue (black arrowhead in B,D). (For non-staining and isotype controls see Appendix 8).

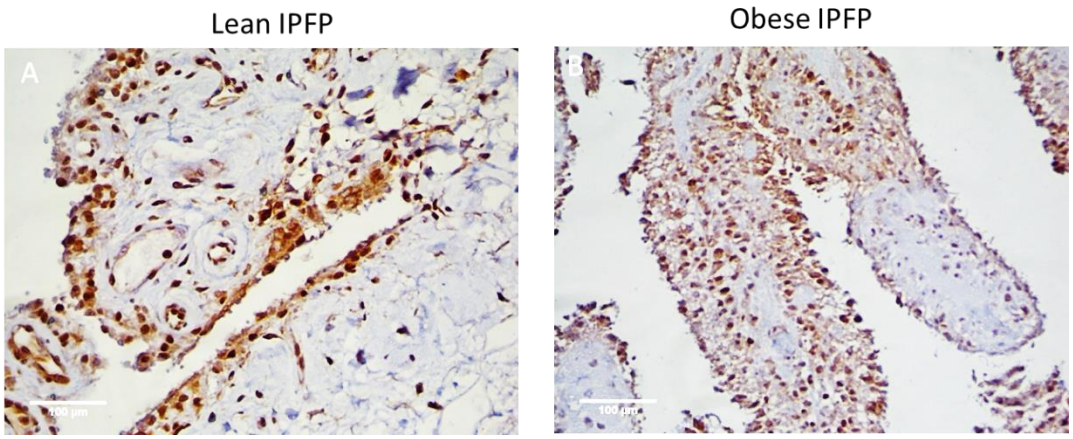



**Figure 5. 12 Visfatin expression in IPFP from Lean and Obese OA patients.**
Representative IHC visfatin staining (brown/black arrows) for IPFP from Lean (A-C) and Obese (D-F) OA patients.

IHC showed abundant visfatin expression in synovial membrane, in adipose and fibrotic tissue as well as in vessels of IPFP (Figure 5.11).

Visfatin is expressed in IPFP of both lean and obese OA patients (Figure 5.12). Western Blot technique was used to analyse the visfatin expression in IPFP tissues protein lysates (Figure 5.13).

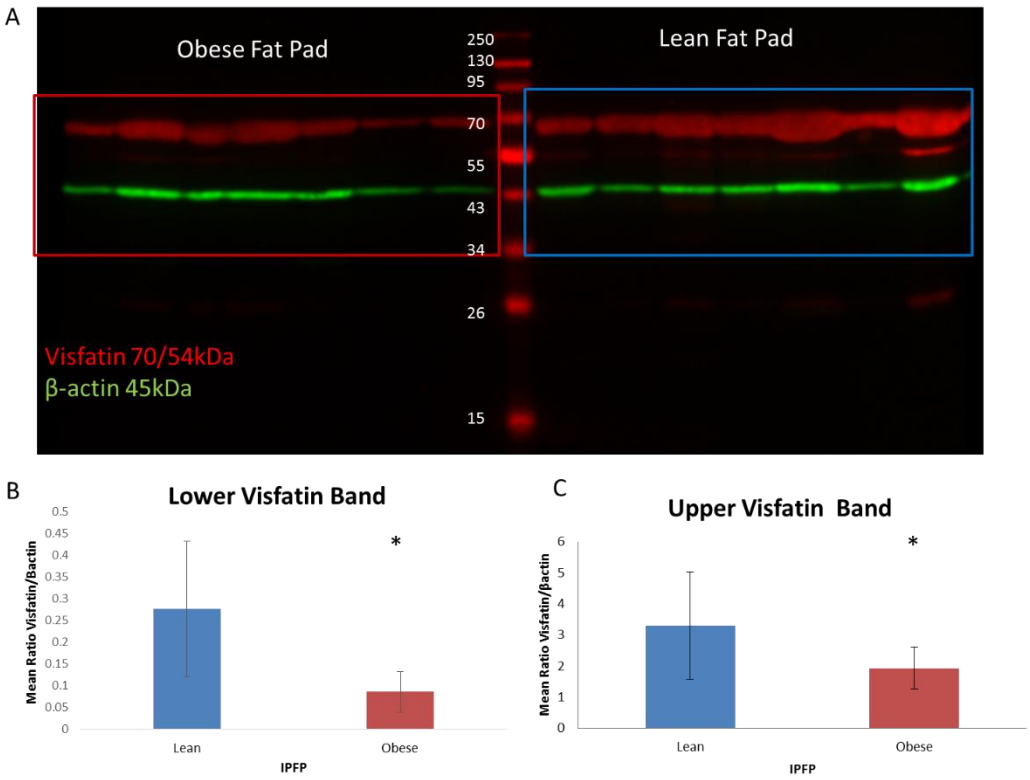



**Figure 5. 13 Semi-quantitative Western blot analysis of visfatin expression in IPFP from Lean and Obese OA patients.**
Representative Western blot result (A) and densitometry analysis (B,C) of visfatin expression in IPFP from Lean and Obese OA patients. Densitometry data are presented as the mean value of the ratio of visfatin to β-actin for each group ± SD (n=10 for each group). p values < 0.05 were considered significant and indicated as *.

Similar to Western Blots of synovial tissue (Chapter 4), two bands were detected, with the lower band significantly fainter that the upper band. Semi-quantitation by densitometry of both bands confirmed the PCR results that visfatin had a significantly lower expression in IPFP from Obese compared to Lean OA patients (Figure 5.13).

## 5.4 Paired comparison of gene and protein expression in synovium and IPFP

Markers including visfatin, PPARγ and adiponectin show differential expression in both synovium and IPFP of obese OA patients. The purpose of this analysis was to investigate the difference between two fatty tissue depots within the knee joint: IPFP and synovium.

### 5.4.1 Visfatin

The difference between synovium and IPFP in NAMPT gene expression is shown in Figure 5.14.

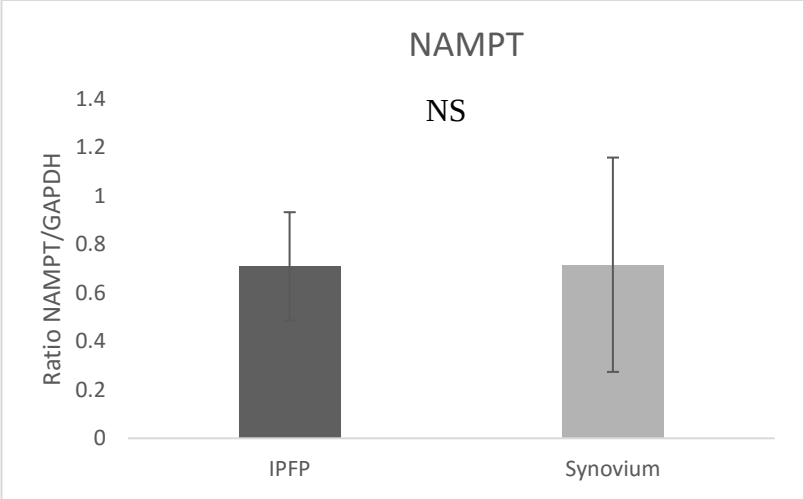



**Figure 5. 14 Semi-quantitative PCR analysis of NAMPT (visfatin) expression in IPFP and synovium obtained from OA patients.**
GAPDH was used as a reference gene. The mean ratio values of each group and standard deviation (SD) bars are shown n=20 for each tissue. NS-non-significant.

Visfatin is expressed at a similar level by the IPFP and the synovium of OA patients (Figure 5.14).

## 5.4.2 Adiponectin

### 5.4.2.1 Semi-quantitative RT-PCR analysis of ADIPOQ gene expression in paired Synovial and IPFP samples

RT-PCR analysis of ADIPOQ gene expression showed that it was significantly lower in synovium as compared to IPFP of OA patients (all analysed tissues) (Figure 5.15) Looking at patient groups based on BMI this was also true for obese patients, whereas lean OA patients showed no significant difference in ADIPOQ expression in IPFP and synovium.

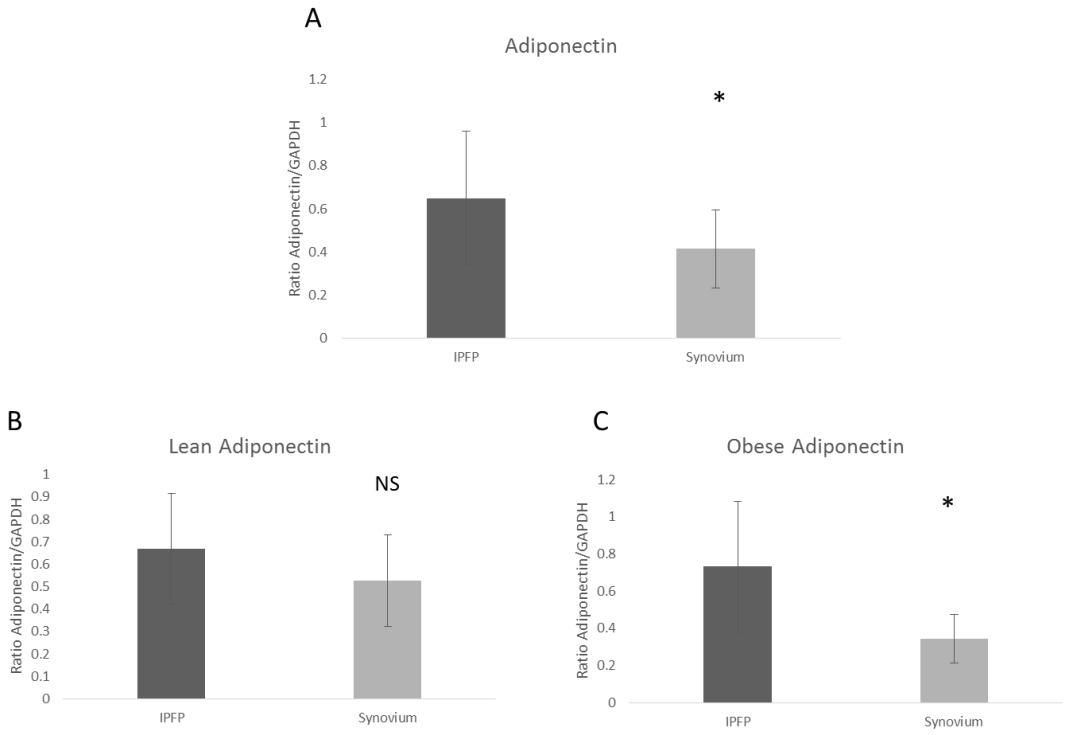



**Figure 5. 15 Semi-quantitative PCR analysis of adiponectin expression in IPFP and synovium obtained from Lean and Obese OA patients.**
GAPDH was used as a reference gene. The mean ratio values of each group and standard deviation (SD) bars are shown for all obtained samples (A) and separated according to BMI into Obese patients (B) and Lean patients (C). p values < 0.05 were considered significant and indicated as *. NS-non-significant.

#### 5.4.2.2 Quantitative Real Time qPCR analysis of ADIPOQ gene expression in paired Synovial and IPFP samples

The difference between synovium and IPFP ADIPOQ gene expression assessed by Real Time qPCR is shown in Figure 5.16

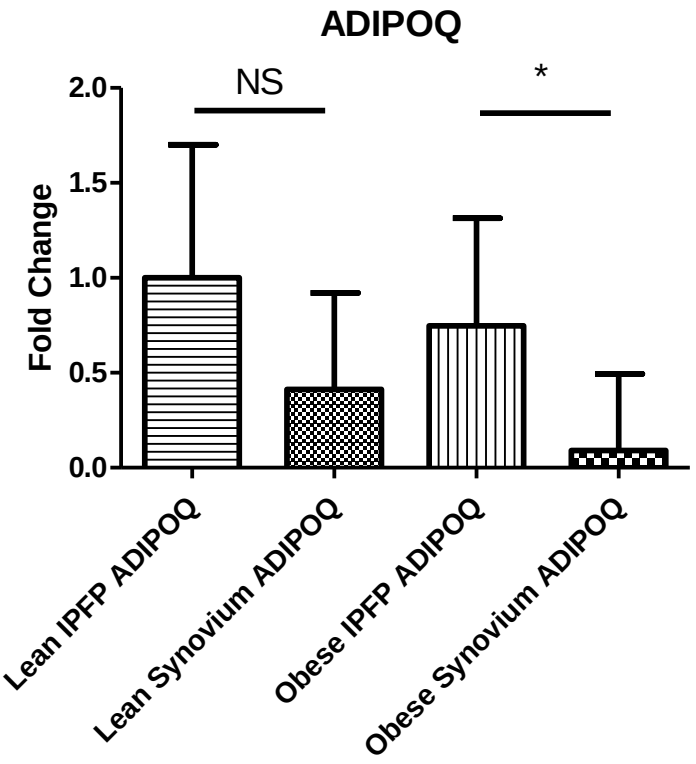



**Figure 5. 16 Quantitative PCR analysis of ADIPOQ expression in IPFP and synovium obtained from Lean and Obese OA patients.**
Quantitative analysis of ADIPOQ gene expression in paired IPFP and synovium from OA patients. B2M was used to normalise gene expression. Groups termed "Lean" n=7 and "Obese" n=9 were analysed. The -ΔΔCT method was used. The mean value of Lean IPFP Group was used as a calibrator to investigate fold change of gene expression. The ratio values of each group and SEM bars are shown. p values < 0.05 were considered significant and indicated as *.NS-not significant.

RT-PCR (Figure 5.15) and quantitative Real Time PCR analysis (Figure 5.16) revealed that adiponectin was significantly higher in IPFP compared to the synovium of all OA patients analysis. Further, paired statistical analysis of RT-PCR and qPCR data showed that the IPFP of Obese patients expressed significantly higher ADIPOQ than synovium from the same patients, while there was no significant difference between tissues in Lean patients.

### 5.4.2.3 Adiponectin protein expression analysis in synovium and IPFP.

The difference between synovium and IPFP adiponectin protein expression was assessed by Western blot and ELISA are shown in Figure 5.17.

#### 5.4.2.3.1 Western Blot analysis

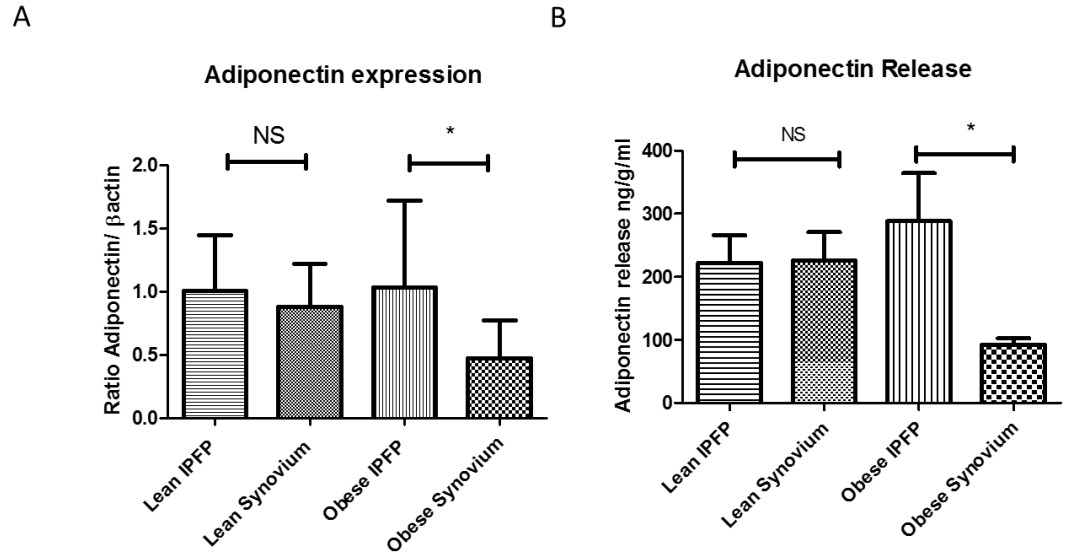



**Figure 5. 17 Semi-quantitative WB analysis of Total Adiponectin expression in IPFP and synovium from Lean and Obese OA patients.**
Densitometry analysis (A) of adiponectin expression in IPFP and synovium in OA patients. Paired Obese and Lean IPFP and synovium ratios are presented. Densitometry data are presented as the mean value of the ratios of adiponectin to β-actin for each group ± SD. Quantification of adiponectin release into media by IPFP and Synovial tissue explants after 3 days in serum free media measured by ELISA (B). Each tissue explant was weighed and cultivated in duplicate. ELISA detection was conducted in duplicates for each sample. Results are shown as mean values ± SD (n=5 for each group). p values < 0.05 were considered significant. NS- non-significant.

Adiponectin protein expression was expressed at a significantly higher level in the IPFP than in the synovium of Obese patients with OA. Also, adiponectin levels in culture supernatants measured by ELISA were significantly higher in the IPFP than in the synovium of Obese IPFP (Figure 5.17).

## 5.4.3 PPARγ

The difference between synovium and IPFP PPARγ gene expression is shown in Figure 5.18-5.19.

### 5.4.3.1 Semi-quantitative RT-PCR analysis of PPARγ gene expression in paired Synovial and IPFP samples

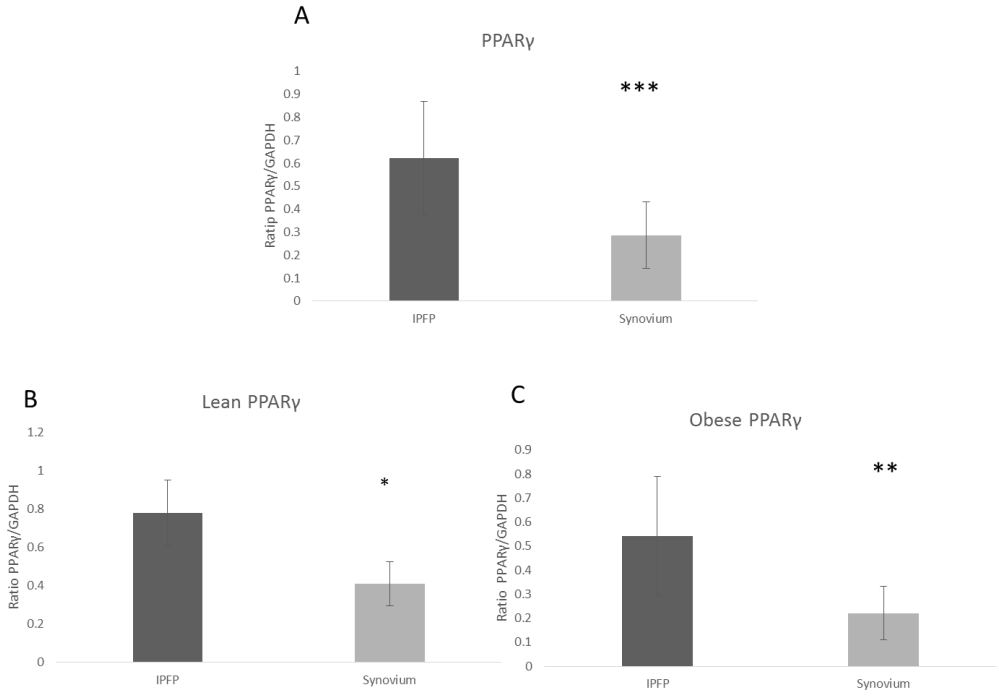



**Figure 5. 18 Semi-quantitative PCR analysis of PPARγ expression in IPFP and synovium from Lean and Obese OA patients.**
GAPDH was used as a reference gene. The mean ratio values of each group and standard deviation (SD) bars are shown for all the samples (A) and separated according to BMI into Obese patients (B) and Lean patients (C). The mean values of each group and standard deviation (SD) bars are shown. p values < 0.05 were considered significant and indicated as *, p value< 0.01 were indicated as **, p value< 0.001 were indicated as ***.

Semi-quantitative PCR analysis showed that PPARγ was significantly lower in synovium as compared to IPFP in all analysed patients and when comparing among the obese and lean groups.

### 5.4.3.2 Quantitative Real time qPCR analysis of PPARγ gene expression in paired Synovial and IPFP samples

The difference between synovium and IPFP PPARγ gene expression assessed by Real Time PCR technique is shown in Figure 5.19

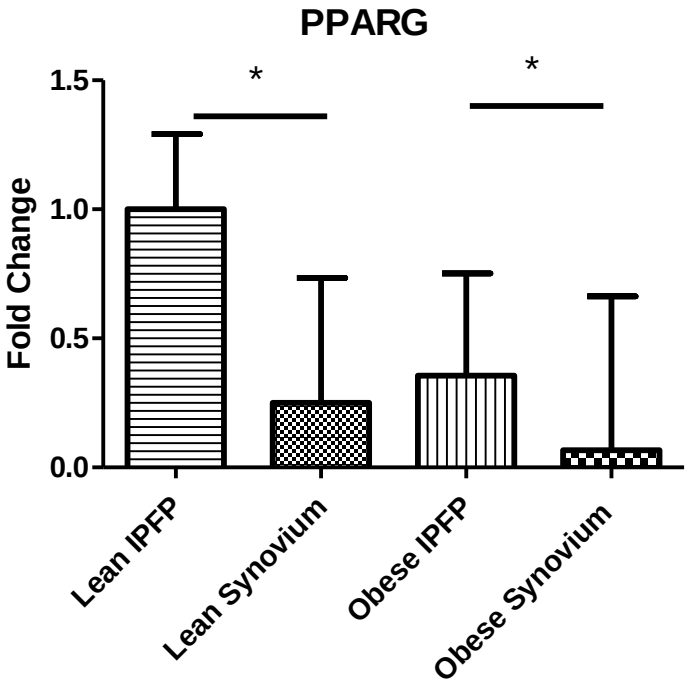



**Figure 5. 19 Quantitative PCR analysis of PPARγ expression in IPFP and synovium obtained from Lean and Obese OA patients.**
Quantitative analysis of PPARγ gene expression in IPFP and synovium from OA patients. B2M was used to normalise gene expression. Groups termed "Lean" n=7 and "Obese" n=9 were analysed.The -ΔΔCT method was used to investigate fold change in gene expression. The mean values of each group and SEM bars are shown. p values < 0.05 were considered significant and indicated as *.

RT-PCR and quantitative Real Time PCR analysis revealed that PPARγ gene was significantly higher in the IPFP compared to the synovium of OA patients. Further paired statistical analysis showed that the IPFP of both Obese and Lean patients expressed PPARγ at a higher level than the synovium from the same patient (Figures 5.18-5.19).

#### 5.4.3.3 PPARγ protein expression analysis in synovium and IPFP

The difference between synovium and IPFP PPARγ protein expression assessed by Western Blot is shown in Figure 5.20.

##### 5.4.3.3.1 Western Blot Analysis

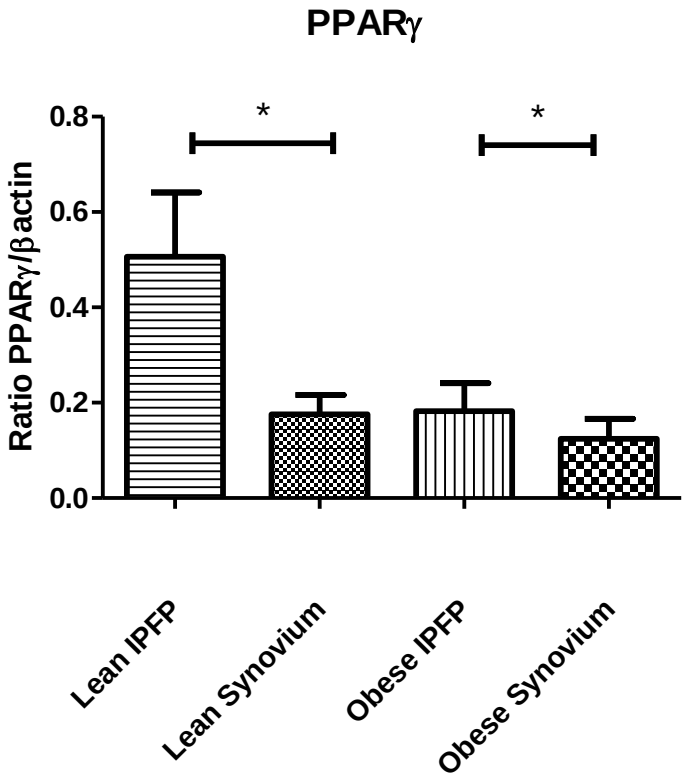



**Figure 5. 20 Semi-quantitative WB analysis of PPARγ expression in IPFP and synovium from Lean and Obese OA patients.**
Densitometry analysis of PPARγ expression in IPFP and synovium in OA patients. The paired IPFP and synovium results for Obese and Lean patients are presented. Densitometry data is present as the mean value of the ratios of PPARγ to β-actin ± SD. p values < 0.05 were considered significant.

PPARγ protein expression was significantly higher in the IPFP compared to the synovium. In addition, in both groups (Lean and Obese) the IPFP expression of PPARγ was significantly higher than in synovium (Figure 5.20).

## 5.5 Summary and Conclusions

In conclusion, obtained data revealed a number of important points:

1) Osteoarthritic Infrapatellar Fat Pad (IPFP) obtained after TKR express obesity relevant markers.
2) IPFP from Obese OA patients expresses adiponectin at the same level as in Lean patients both at mRNA and protein level.
3) IPFP from Obese OA patients expresses PPARγ and visfatin at a lower level than from Lean ones both at mRNA and protein level.
4) Obese synovium express the significantly lower level of adiponectin in comparison to IPFP.
5) IPFP from the same donor expresses significantly higher level of PPARγ than synovium.

All of the above data could help to explain the correlation between obesity and OA. The genes and proteins investigated in this study suggest different molecular pathways in the IPFP from obese and lean individuals. In particular, differences in adiponectin, PPARγ and visfatin expression in IPFP were found to be altered and need to be investigated further. Furthermore, presented data also suggest differences in expression of adiponectin and PPARγ between synovium and IPFP.

# Chapter 6: Regional difference in immune cell population in synovium and IPFP from OA patients

## 6.1 Introduction

Adipose tissue is a highly heterogenic tissue. It consists not only of large vacuolar adipocytes but also progenitor cells (MSCs, preadipocytes), endothelial and vascular cells in addition to cells of the immune system including macrophages, T cells, and mast cells. All of these have a role in adipose tissue homeostasis. In individuals of normal-weight, macrophages phagocyte dying adipocytes maintaining the proper tissue organisation. With weight gain, there is a reorganisation of the whole adipose tissue. Previously protective cells may become more pro-inflammatory. In addition, there is an influx of other cells, which can trigger inflammation of the whole tissue (Sun et al., 2012). In this study, the difference in immune cell populations between lean and obese patients suffering from OA in two different fatty tissue depots: synovium and IPFP were investigated.

Macrophages originate from blood monocytes and are specialised in the phagocytosis of pathogens, tissue debris and apoptotic cells in addition to the repair of wounded tissues (Wynn et al., 2016). Because of their different roles macrophages display high heterogeneity. Initially, macrophages were divided into two main subpopulations M1 and M2 following the lymphocyte T terminology (Th1 and Th2) (Italiani et al., 2015).

M1 macrophage phenotype refers to the “classically-activated” macrophages that manifest their activity during typical pro-inflammatory responses for example microbial invasion. They are activated by IFN-γ and LPS and become potent producers of pro-inflammatory cytokines including TNF-α, CCL-2 and iNOS (Mantovani et al., 2004). Their main role is to eliminate the pathogen and stimulate polarisation of Th1 $CD4^+$ lymphocytes (Murray et al., 2011). M2 so-called

"alternatively-activated" macrophages down-regulate the initial pro-inflammatory activity and initiate tissue repair. They are considered to be anti-inflammatory and mainly activated by IL-4 and IL-14. M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-β (Mantovani et al., 2002). M2 macrophages are also important as the first line of defence against extracellular pathogens for instance parasites and helminths (Mantovani et al., 2005).

The majority of the initial studies on macrophages in obesity were based on murine models in which M1 and M2 subpopulation of macrophages are well characterised. In mice, the phenotype of M2 macrophages is characterised by expressing $CD206^+$ but not $CD11c^-$, while M1 macrophages are $CD206^-$ and $CD11c^+$. When the M1 phenotype is blocked in obese mice there is protection from obesity. In diet-induced obesity in these animals the percentage of $CD11c^+$ macrophages increases by 65-fold (Weisberg et al., 2003).

Unfortunately, animal models do not fully mimic human adipose tissue leukocyte content. More and more studies reveal that the strict M1/M2 terminology described in mice may not reflect the macrophage population in humans. There are different studies defining M1 and M2 markers on human adipose tissue macrophages (ATM) but there is no consensus on the final M1/M2 macrophage profile (Zeyda et al., 2007). Recent studies show that human ATM express a range of both pro- and anti-inflammatory markers (Aron-Wisnewsky et al., 2009; Wentworth et al., 2010). According to Bourlier et al., the majority of ATM express $CD45^+/CD14^+/CD206^+/CD16^-$ and their percentage correlates positively with BMI index. In addition, ATM express MMP-9, which is a remodelling enzyme that can control both adipo- and angiogenesis. The authors speculate that a small population of $CD45^+/CD14^+/CD206^+/CD16^+$ macrophages are also found within adipose tissue and reflect the resident macrophage population while $CD45^+/CD14^+/CD206^+/CD16^-$ macrophages may be influx macrophages (Bourlier et al., 2008).

Besides, macrophages a range of other leukocytes has been investigated. Other representatives of innate immune cells including eosinophils have been identified in adipose tissue and their number is negatively correlated with weight gain (Huh et al.,

2014). They are an important source of anti-inflammatory factors for example IL-4 in adipose tissue from lean individuals. Mast cells content increases with obesity and pro-inflammatory factors such as IL-6 and IFN-γ produced by mast cells can contribute to pro-inflammatory conditions in obesity (Liu et al., 2009).

The role of cells of the adaptive immune system in obesity is also a matter of investigation. B cells, which in lean adipose tissue provide protection from infection, with weight gain increase in number and change their profile to a more pro-inflammatory phenotype.

T cells defined as $CD3^+$ have been reported to be up-regulated in obesity (O'Rourke et al., 2013). Both $CD4^+$ (Winer et al., 2009) and $CD8^+$ T-cells (Nishimura et al., 2009) have been detected in human adipose tissue. However, some studies have reported that the subset of $CD4^+$cells is reduced, whereas the $CD8^+$ number increased in human obese adipose tissue. Whilst other studies, in a mouse model, have reported that both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells number increased in diet-induced obesity (Deiuliis et al., 2011). Increased T lymphocyte numbers may trigger macrophage influx (Nishimura et al., 2009). All of the reported studies propose that immune cell content is crucial in the pathogenesis of obesity-related inflammation.

### 6.1.1 Aims of the study:

The goal of the presented study was to compare differences in leukocytes markers expression in synovium and IPFP of patients undergoing knee replacement surgery.

Specifically, the aims were

- To ascertain if there is a difference in immune cell content in articular adipose tissues depots in obese and lean patients with OA.

# 6.2 Results

## 6.2.1 Macrophages in articular adipose tissues

The presence of macrophages in both synovium and IPFP was investigated using surface marker expression analysis with a Flow Cytometry technique. The strategy for gating is presented in Appendix 5. Figure 6.1-Figure 6.12 show the difference in expression of macrophage markers in synovium and IPFP from lean and obese OA patients.

### 6.2.1.1 $CD45^+$ $CD14^+$ positive cells

Two soft tissue biopsies were harvested from different compartments of the knee joint of each patient: synovial explant and IPFP. Patients were divided into two groups (Lean and Obese) as previously described. Histological analysis did not show any differences in synovitis score between the obese and lean groups (The representative histology H&E see Appendix 8). Each explant consisted of the synovial layer and the majority of the supporting fatty tissue layer.

Example of $CD45^+$ $CD14^+$ gating are shown in Figure 6.1:

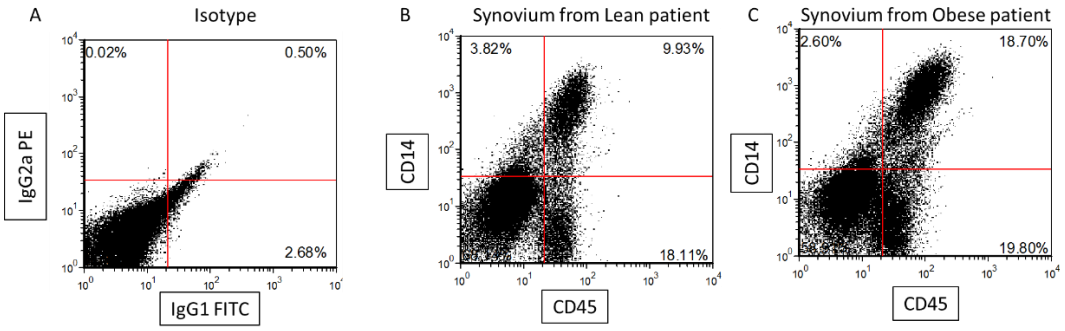
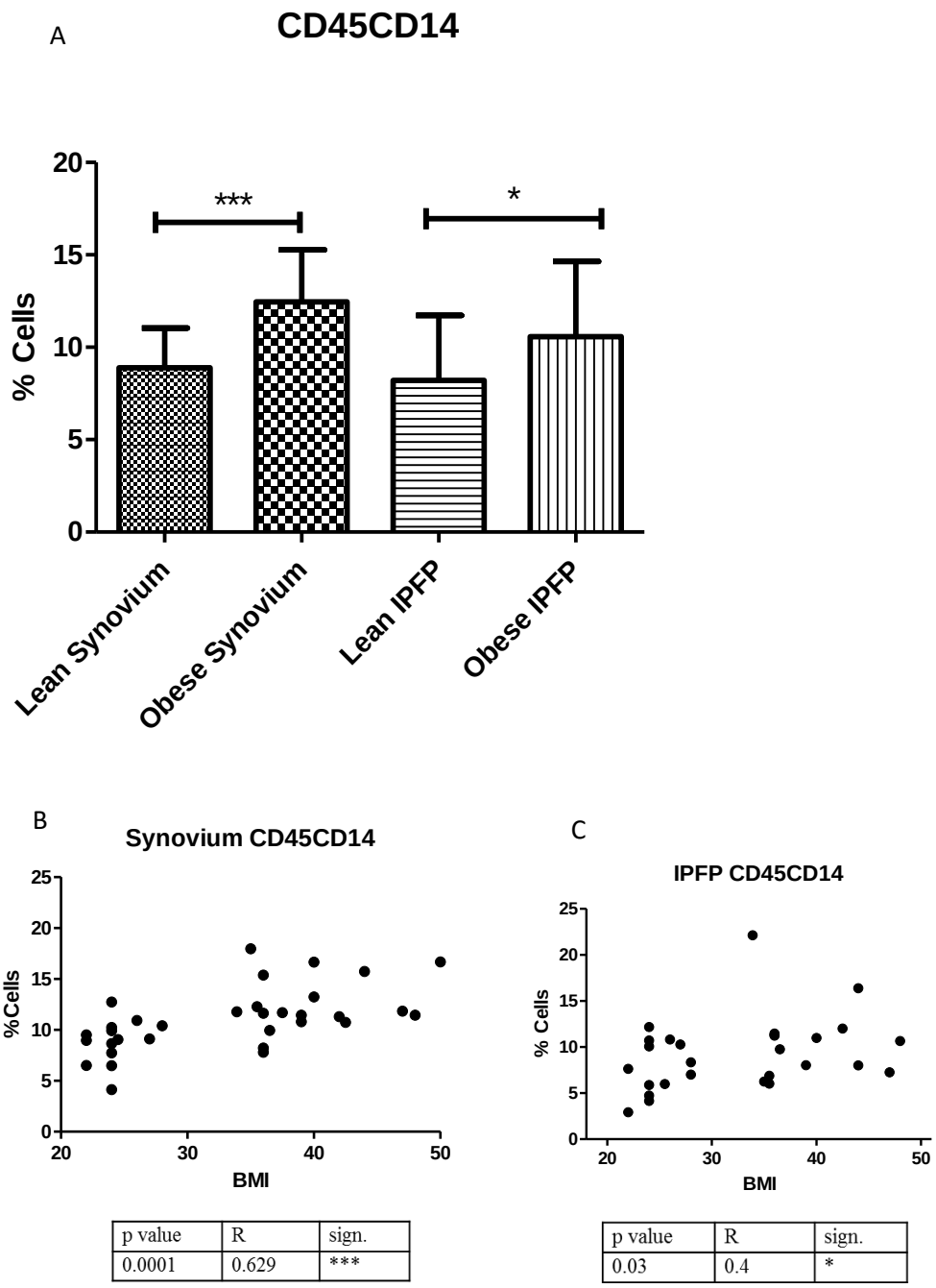



**Figure 6. 1 $CD45^+CD14^+$ macrophage detection in synovium and IPFP in stromovascular fraction (SVF).**
SVF fractions were analysed according to CD45 and CD14 marker expression. The isotype control (A) was analysed at the same time with the sample of interest. Representative plots for Lean (B) and Obese (C) Synovial SVF staining is presented**.**

The percentage of $CD45^+CD14^+$ positive cells in the total cell population (called here SVF) was calculated (see Figure 6.2).




| p value | R | sign. |
|---|---|---|
| 0.0001 | 0.629 | *** |

| p value | R | sign. |
|---|---|---|
| 0.03 | 0.4 | * |

**Figure 6. 2 Content of $CD45^{+}CD14^{+}$cells in SVF fraction of synovium and IPFP from Lean and Obese OA patients.**
The mean percentage for the group termed "Lean" n=14 for synovium n=13 for IPFP and "Obese" n=19 for synovium n=14 for IPFP ±SD was shown (A). p value <0.05 indicated as *, p value<0.005 indicated as ***.Correlation of percentage of $CD14^{+}CD45^{+}$ with BMI score in synovium and IPFP (B, C).

The data (Figure 6.2) suggest that there was a higher frequency of macrophages in the synovium and IPFP of obese OA patients in comparison to lean ones. There was no difference in the macrophage content between paired fatty depots within the joint (synovium vs. IPFP) but there was a significant correlation of macrophage percentage and BMI score both in synovium and IPFP. MFI analysis showed no difference in intensity of CD14 and CD45 staining between all groups (data not shown).

#### 6.2.1.2 $CD14^+$ $CD206^+$ positive cells

Another reported macrophage marker CD206 has been investigated (see Figure 6.3).

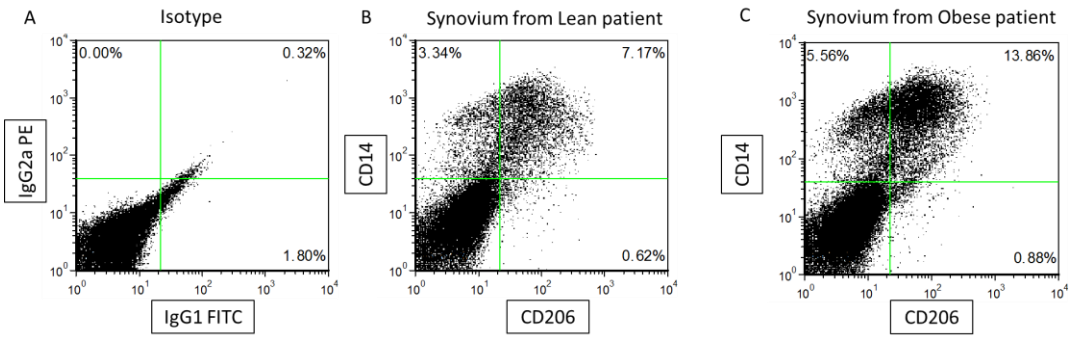
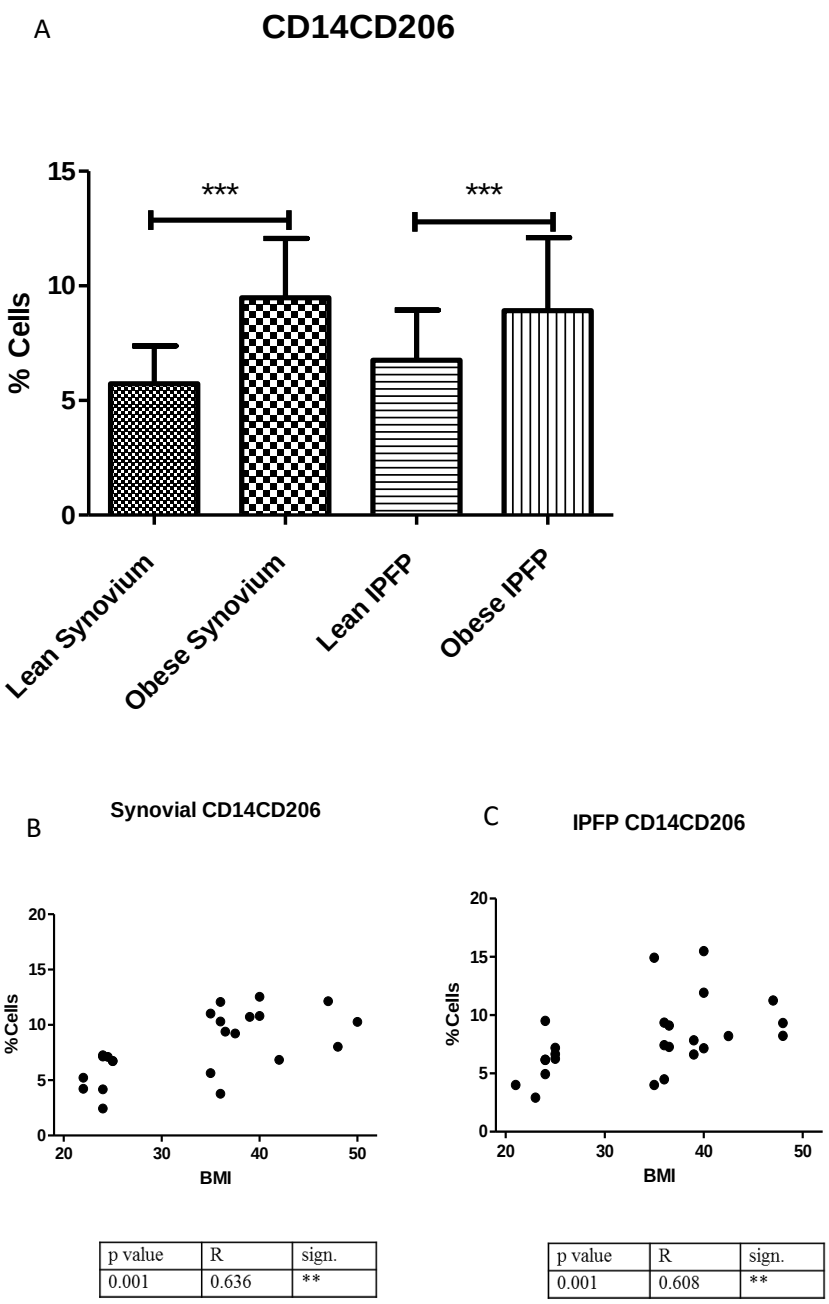



**Figure 6. 3 $CD14^+CD206^+$ macrophage marker detection in synovium and IPFP SVF fraction.** SVF fractions were analysed for CD14 and CD206 expression. Isotype control (A) was analysed at the same time as a sample of interest. Representative plots for Lean (B) and Obese (C) SVF staining are presented.

The percentage of $CD14^+CD206^+$ positive cells in the SVF cell population was calculated (see Figure 6.4).




| p value | R | sign. |
|---|---|---|
| 0.001 | 0.636 | ** |

| p value | R | sign. |
|---|---|---|
| 0.001 | 0.608 | ** |

**Figure 6. 4 Percentage of CD14+CD206+ in SVF fraction of synovium and IPFP from Lean and Obese OA patients.**
The mean percentage for the group termed "Lean" n=8 and "Obese" n=14 for synovium, n=16 for IPFP ±SD is shown (A). p value<0.001 indicated as ***The correlation of the percentage of CD14+CD206+ with BMI score in the synovium and IPFP is shown in (B) and (C) respectively.

The data (Figure 6.4) suggest that there was a higher frequency of $CD14^+$ $CD206^+$ cells in the synovium and IPFP of Obese OA patients in comparison to lean ones. There was no difference in $CD14^+CD206^+$ cell content between synovium vs. IPFP

from the same joint. MFI analysis showed no difference in intensity of CD14 and CD206 staining between all groups (data not shown).

#### 6.2.1.3 CD86 CD14 positive cells

M1 macrophage marker CD86 was also analysed (see Figure 6.5).

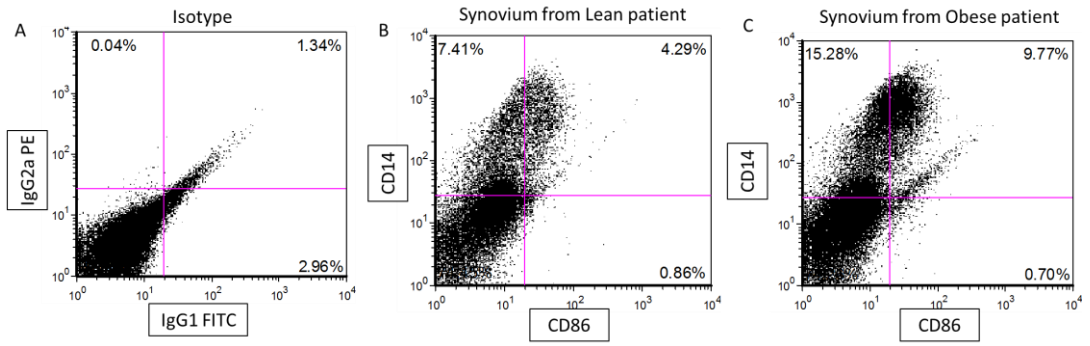
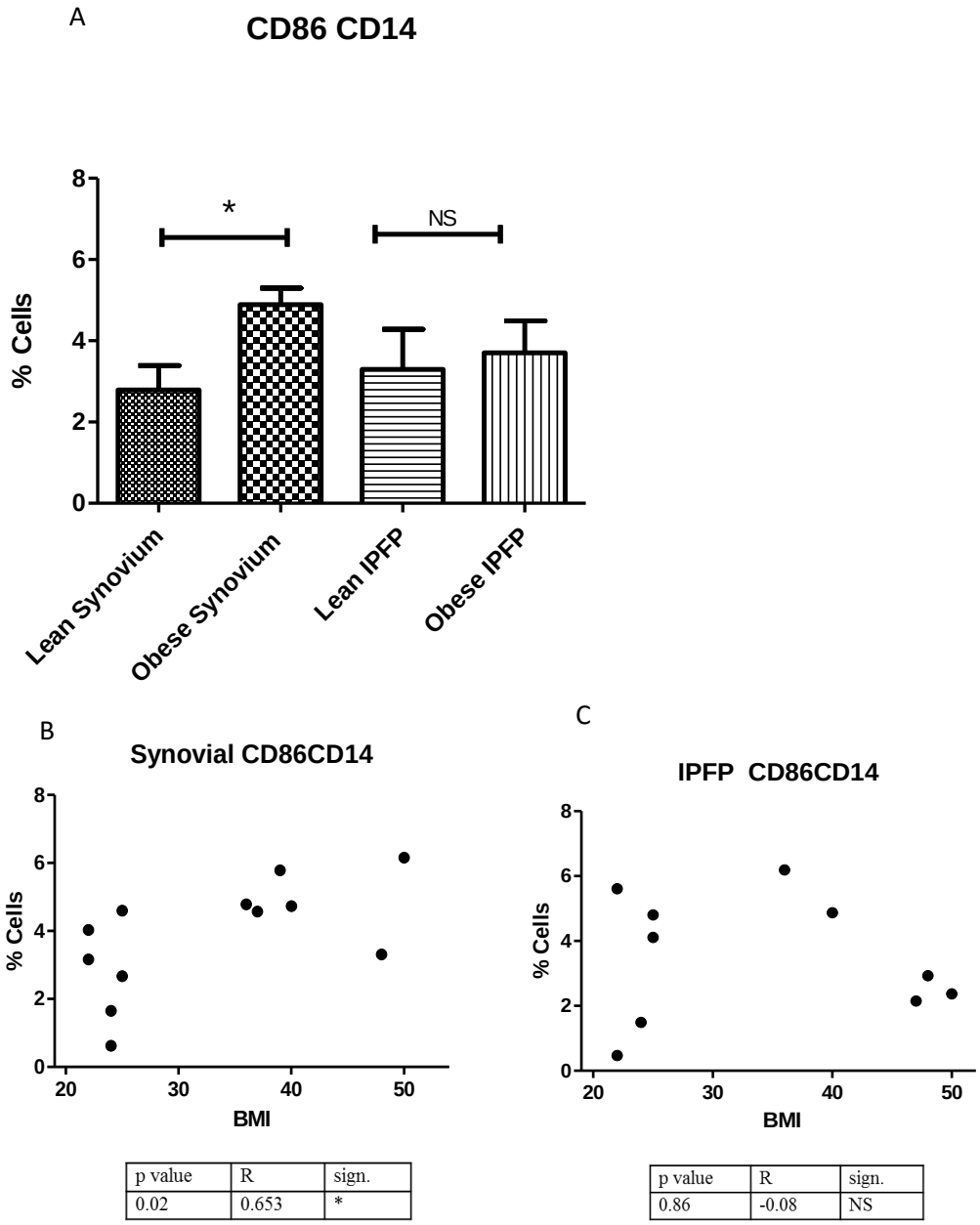



**Figure 6. 5 $CD86^{+}CD14^{+}$ macrophage markers detection in synovium and IPFP SVF fraction**. SVF fractions were analysed for CD14 and CD86 expression. Isotype control (A) was analysed at the same time with sample of interest. Representative plots for Lean (B) and Obese (C) SVF of synovium staining is presented.

The percentage of $CD86^{+}CD14^{+}$ positive cells in the SVF cell population was calculated (see Figure 6.6).




| p value | R | sign. |
|---|---|---|
| 0.02 | 0.653 | * |

| p value | R | sign. |
|---|---|---|
| 0.86 | -0.08 | NS |

**Figure 6. 6 Percentage of CD86+CD14+ in SVF fraction of synovium and IPFP from Lean and Obese OA patients (A**).
The mean percentage for the group termed as "Lean" n=7 and "Obese" n=6±SD is shown (A). The correlation of the percentage of CD86+CD14+ with BMI score in the synovium and IPFP is shown in (B) and (C) respectively p value<0.05 indicated as *

The data obtained (Figure 6.6) suggest that there was a higher frequency of $CD14^{+}$ $CD86^{+}$ cells in the synovium of obese OA patients in comparison to Lean ones, but there was no difference in IPFP between patient groups. There was no difference

between paired synovium and IPFP in $CD86^{+}CD14^{+}$ frequency. MFI analysis showed no difference in intensity of CD14 and CD86 staining between all groups (data not shown).

### 6.2.1.4 Other macrophage markers: HLA-DR

Another macrophage-related marker HLA-DR has been investigated (see Figure 6.7).

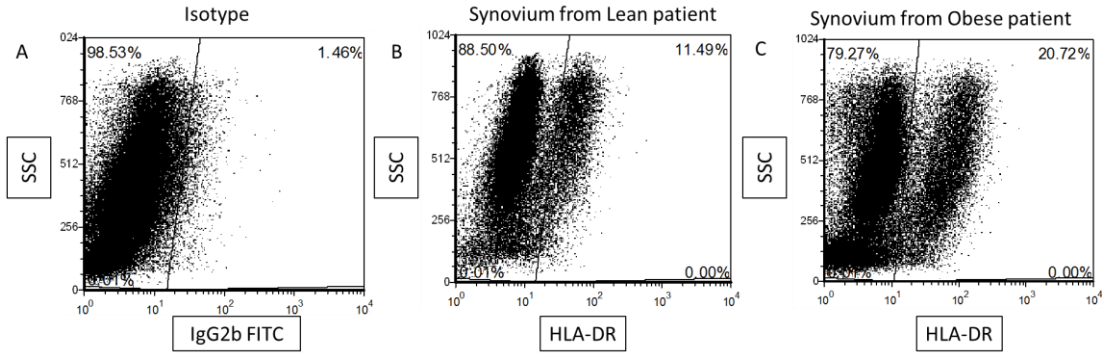
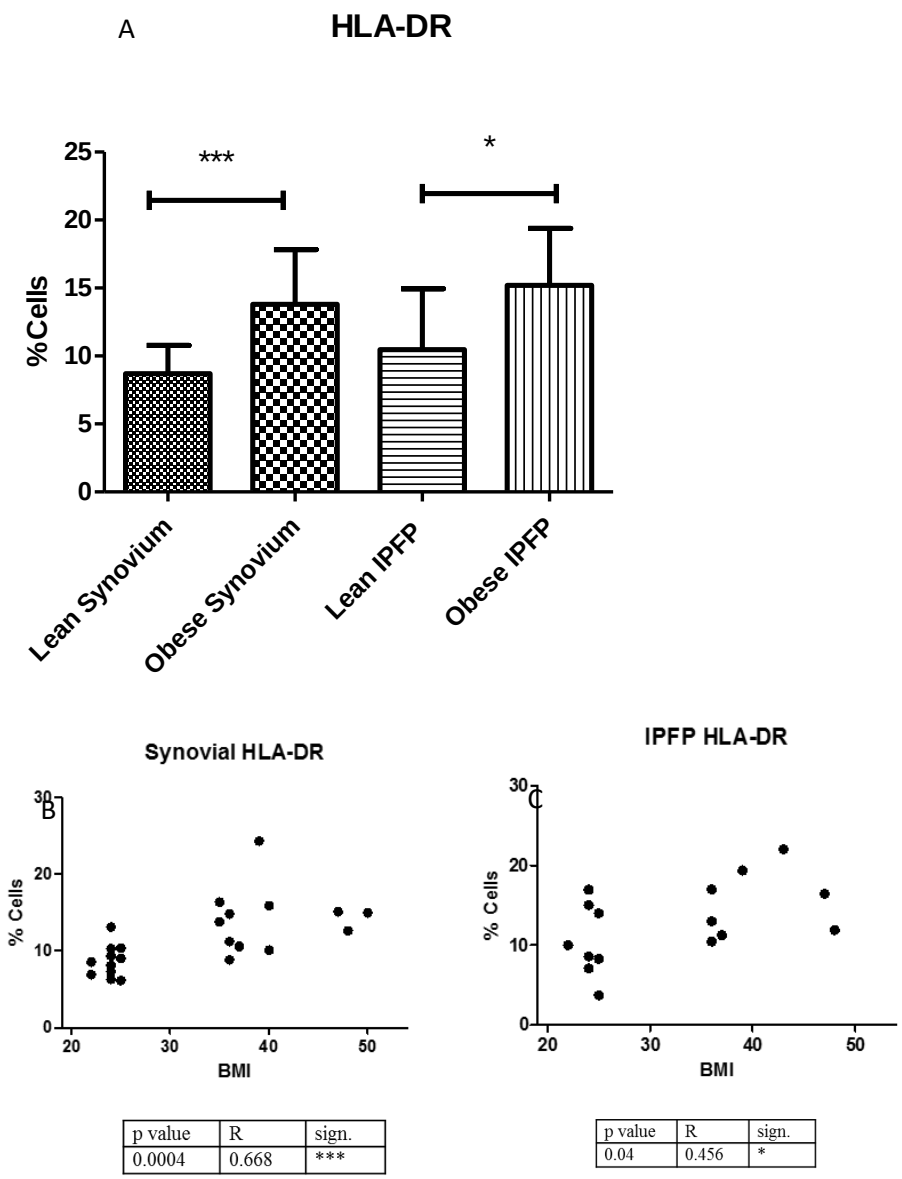



**Figure 6. 7 HLA-DR markers detection in synovium and IPFP SVF fraction.**
SVF fractions were analysed according to HLA-DR expression. Isotype control (A) was analysed at the same time with a sample of interest. Representative plots for Lean (B) and Obese (C) SVF staining are presented.

The percentage of HLA-DR positive cells in the SVF cell population was calculated (see Figure 6.8).




| p value | R | sign. |
|---|---|---|
| 0.0004 | 0.668 | *** |

| p value | R | sign. |
|---|---|---|
| 0.04 | 0.456 | * |

**Figure 6. 8 Percentage of HLA-DR$^+$ cells in SVF fraction of synovium and IPFP from Lean and Obese OA patients.**
The mean percentage for the group termed "Lean" n=12 for synovium, n=8 for IPFP and "Obese" n=12 for synovium, n=8 for IPFP ±SD is shown (A, B). The correlation of the percentage of HLA-DR$^+$ with BMI score in the synovium and IPFP is shown in (B) and (C) respectively p value<0.05 indicated as *, p value<0.001 indicated as ***.

The data (Figure 6.8) suggest that there was a higher frequency of HLA-DR$^+$ cells in both the synovium and IPFP from obese OA patients in comparison to lean ones. There was no difference between paired synovium vs. IPFP HLA-DR$^+$ cell percentage in both groups.

HLA-DR mean fluorescence intensity was also analysed. MFI analysis didn't show the difference in HLA-DR expression indicating that the difference between lean and obese samples comes from increased number of HLA-DR$^+$ cells but not the increased expression of that protein on the surface (data not shown).

### 6.2.1.5 CD36

CD36 expression was also analysed (see Figure 6.9).

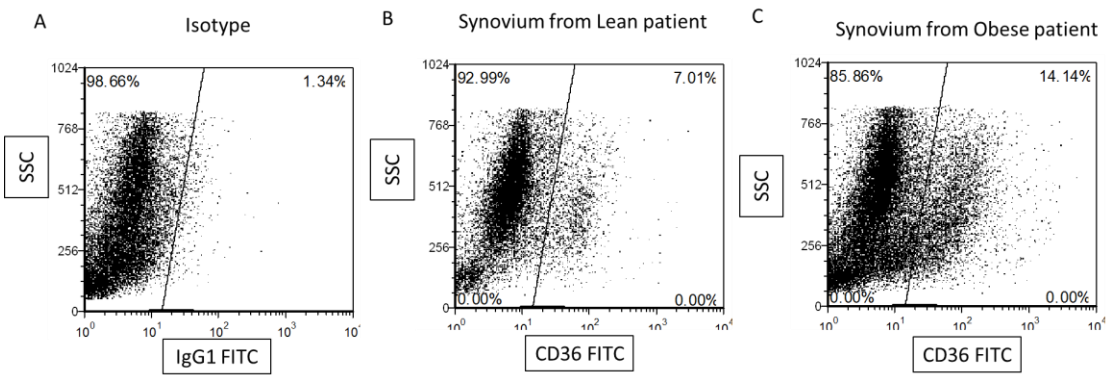



**Figure 6. 9 CD36$^{+}$ markers detection in synovium and IPFP SVF fraction.**
SVF fractions were analysed according to CD36$^{+}$ expression. Isotype control (A) was analysed at the same time with a sample of interest. Representative plots for Lean (B) and Obese (C) SVF staining is presented.

The percentage of CD36-positive cells in the SVF cell population was calculated (see Figure 6.10).

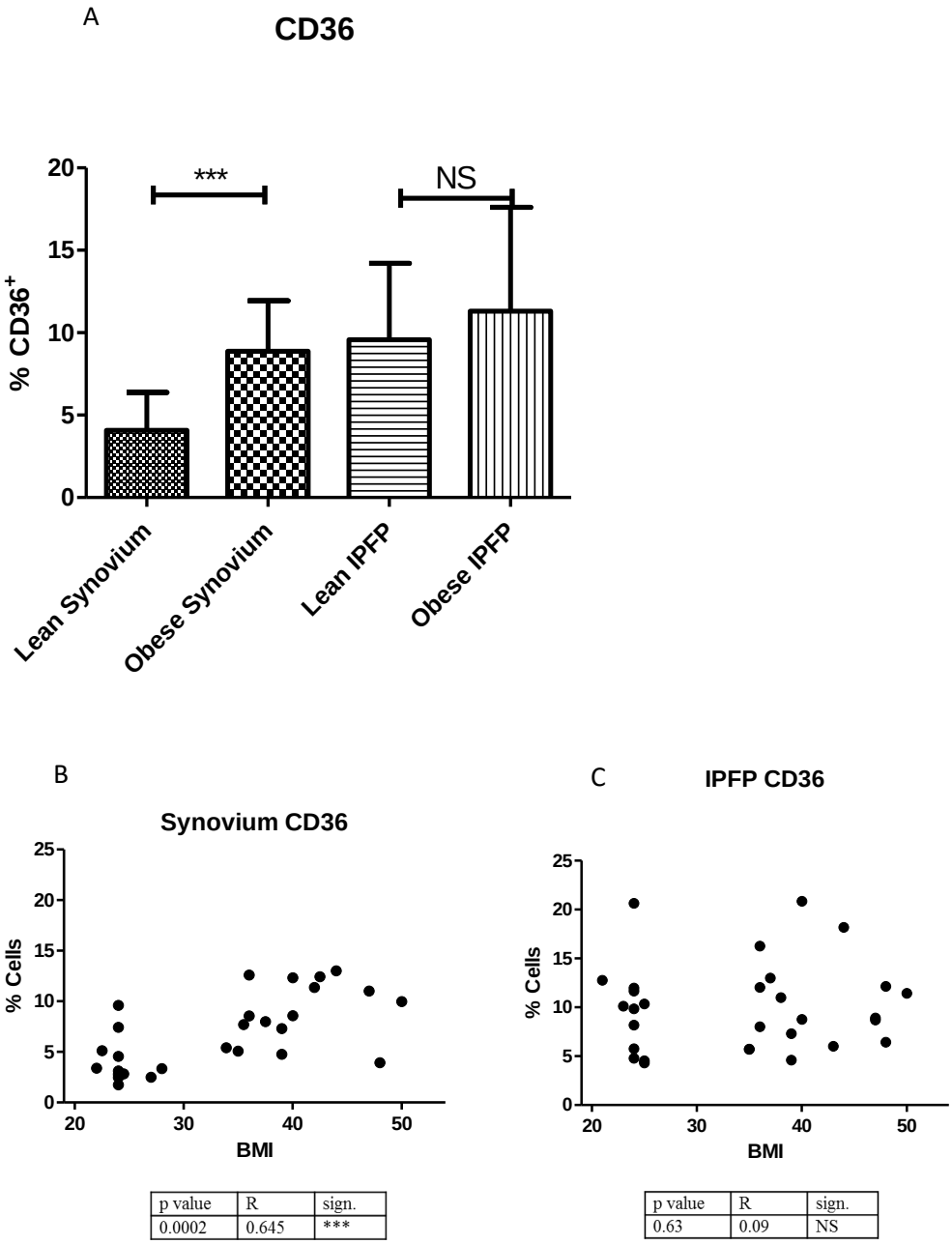



**Figure 6. 10 Percentage of CD36$^+$ cells in SVF fraction of synovium and IPFP from Lean and Obese OA patients (A).**
The mean percentage for the group termed "Lean" n=12 and "Obese" n=16 ±SD is shown (A)The correlation of the percentage of CD36+ with BMI score in the synovium and IPFP is shown in (B) and (C) respectively. p value<0.001 indicated as ***.

The data (Figure 6.11) suggest that there was a higher frequency of CD36$^+$ cells in the synovium from Obese OA patients in comparison to Lean ones. There was no

difference in $CD36^+$ cell frequency in the IPFP of obese and lean patients, but there was a significant difference between the paired synovium vs. IPFP $CD36^+$ cell content.

MFI analysis didn't show the difference in CD36 expression indicating that the difference between lean and obese samples comes from increased number of $CD36^+$ cells but not the increased expression of that protein on the surface.

## 6.2.2 T lymphocytes

Two soft tissue were also analysed for T lymphocyte content. The gating strategy is shown in Figure 6.11.

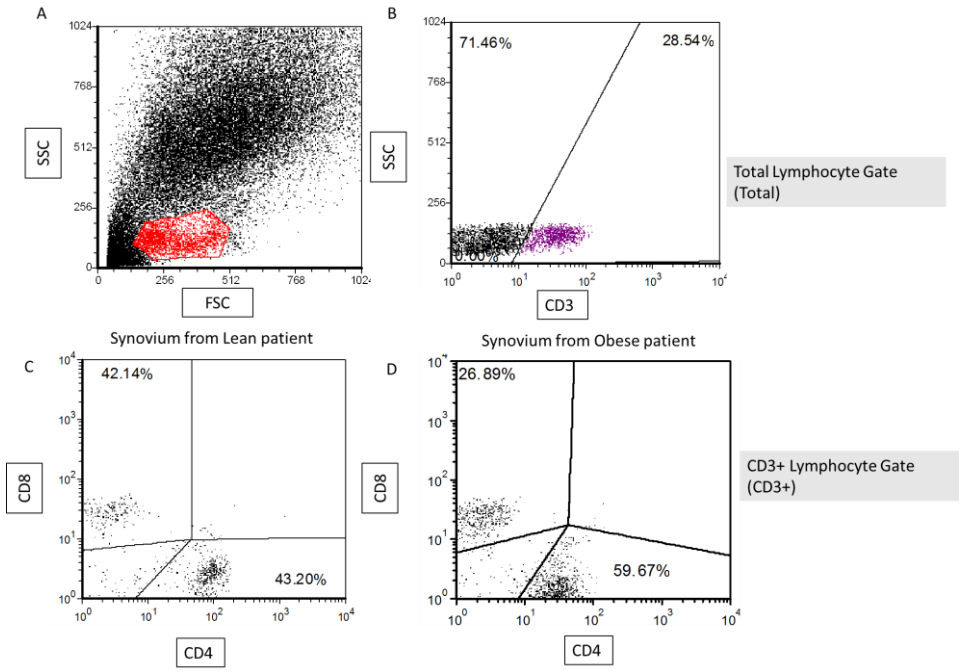



**Figure 6. 11 T cells detection in synovium SVF fraction.**
Gating strategy (A, B, C) and representative plots for CD3, CD3CD4 and CD3CD8 cells detection. Percentage of T cells subpopulations in SVF fraction of synovium from Lean and Obese OA patients (D).

The percentage of T cell subpopulation in synovial SVF is shown in Figure 6.12.

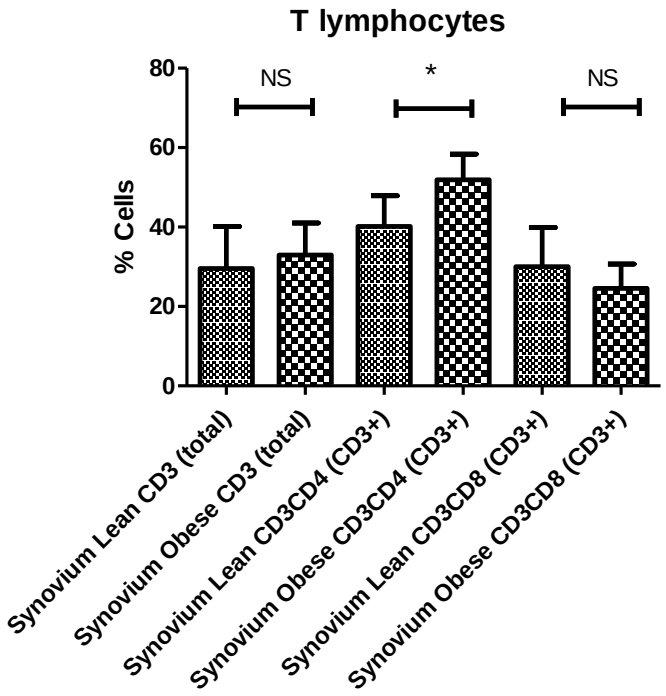



**Figure 6. 12 T cells detection in synovium SVF fraction**.
Percentage of T cells subpopulations in SVF fraction of synovium from Lean n=6 and Obese n=10 OA patients. The mean percentage for the group ±SD is shown. p value<0.05 indicated as *.

The data obtained here (Figure 6.12) suggest that there was no difference in the frequency of T cells (CD3 positive cells) in the synovium of obese patients with OA in comparison to lean ones. The frequency of CD3 CD4 positive cells was significantly higher in obese OA patients in the synovium. The CD3 CD8 positive T cell frequency was not different between the lean and obese groups.

#### 6.2.2.1.1 IPFP T lymphocytes

The percentage of T cell subpopulation in IPFP SVF is shown in Figure 6.13.

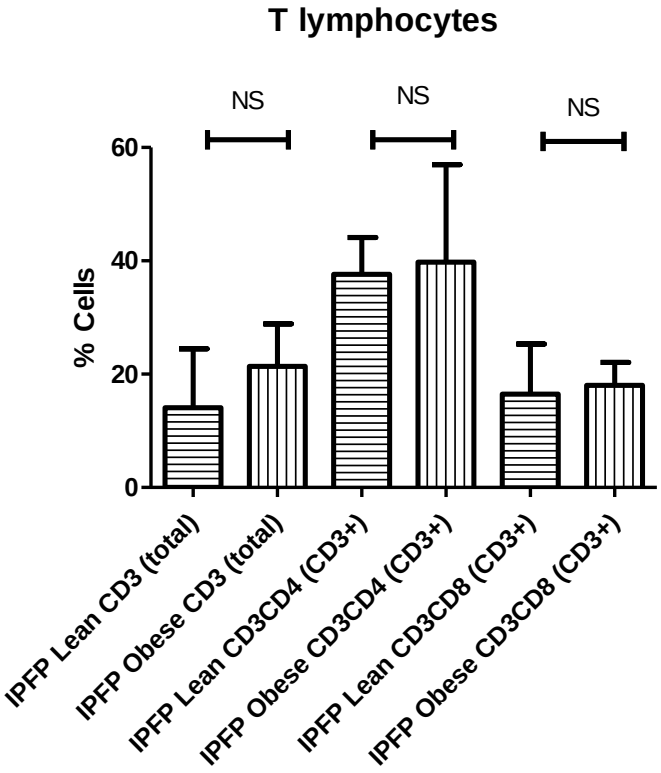



**Figure 6. 13 T cells detection in IPFP SVF fraction**.
Percentage of T cells subpopulations in SVF fraction of IPFP from Lean n=6 and Obese n=10 OA patients. The mean percentage for the group ±SD is shown. p value<0.05 indicated as *, NS-non-significant.

The data (Figure 6.13) suggest that there was a tendency towards a higher frequency of CD3 positive cells in the IPFP (p=0.08) of obese patients with OA but this difference was not statistically significant. The frequency of CD3 CD4 and CD3 CD8 positive cells was not different between obese OA patients from the two groups.

#### 6.2.2.1.2 T cell population analysis in IPFP versus synovium

Differences in synovium and IPFP content of CD3, CD3CD4 and CD3CD8 positive cells was also investigated (Figure 6.14).

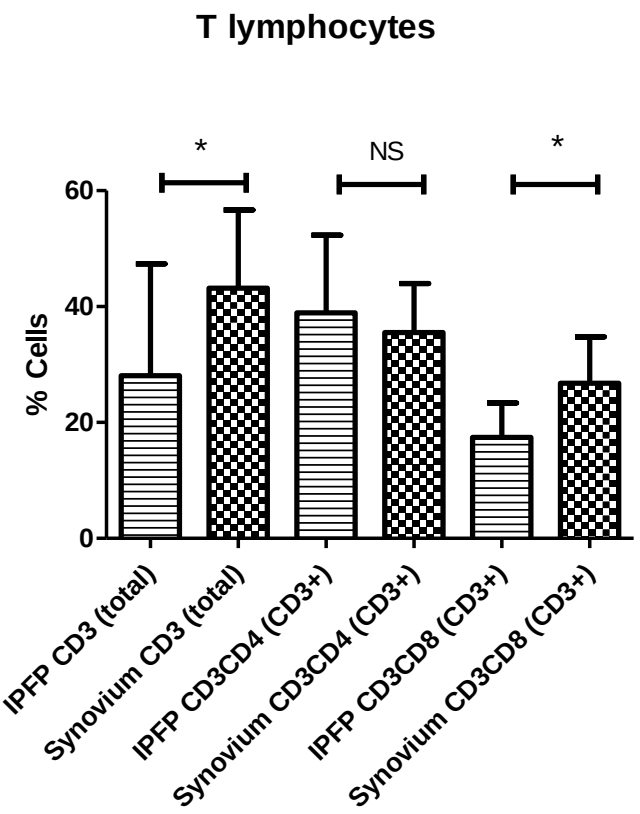



**Figure 6. 14 T cells detected in synovium and IPFP SVF**.
Percentage of T cells subpopulations in SVF fraction of synovium and IPFP from OA patients. The mean percentage for the group ±SD is shown. p value<0.05 indicated as *.

The data presented (Figure 6.14) suggest that there was a significantly higher frequency of CD3 positive cells and CD3CD8 in the synovium compared to the IPFP while there was no difference in the CD3CD4 population between IPFP and synovium from the same donor.

T cell subtypes (CD4/CD8) ratio has been postulated to be important in several tissues. Differences in synovium and IPFP content of ratio of CD3CD4 to CD3CD8 was also investigated (Figure 6.15).

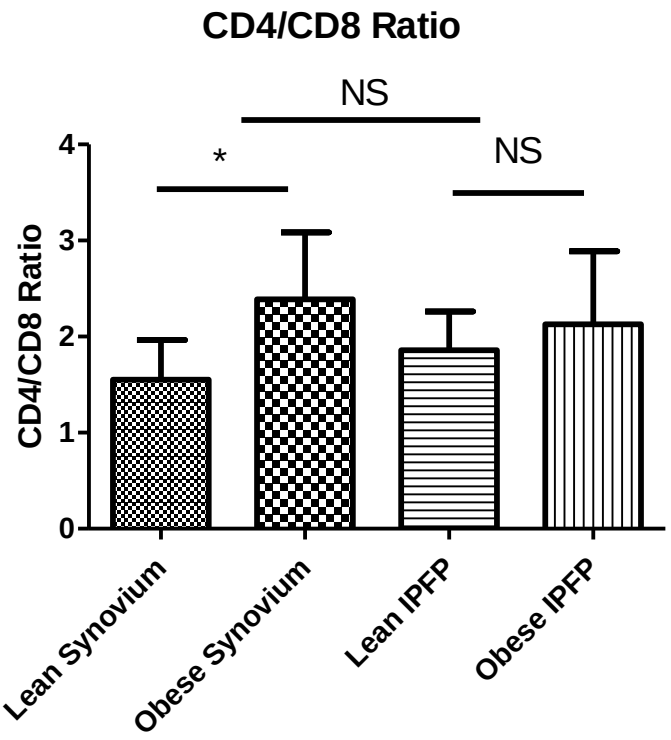



**Figure 6. 15 CD4/CD8 T cell ratio detected in synovium and IPFP SVF**.
The ratio of the percentage of T cells subpopulations in SVF fraction of synovium and IPFP from OA patients. The mean ratio of the percentage CD3CD4/CD3CD8 for each of the group ±SD is shown. p value<0.05 indicated as *.ns – non-significant.

Data suggest that there is the significantly higher ratio of CD3CD4/CD3CD8 T cells in synovium from Obese in comparison to Lean OA patients and there is no difference in IPFP in both groups. Furthermore, there is no difference in CD3CD4/CD3CD8 ration between paired synovium and IPFP.

## 6.3 Summary and Conclusions

In conclusion, obtained data revealed a number of important points:

1) Osteoarthritic Infrapatellar Fat Pad (IPFP) and synovium obtained after TKR are potent sources of adipose tissue macrophages (ATM).
2) IPFP and synovium from Obese OA patients have an increased number of Adipose Tissue Macrophages characterized by $CD45^{+}CD14^{+}$, $CD14^{+}CD206^{+}$. Furthermore, synovium from obese patients is characterized by increased number of $CD86^{+}CD14^{+}$ cells.
3) Increase number of ATMs in synovium and IPFP is accompanied with increase expression of other macrophage-related proteins (e.i HLA-DR).
4) Lean synovium express significantly lower CD36 in comparison to paired IPFP. CD36 expression increases in the synovium of obese patients in comparison to lean ones and doesn't change in IPFP.
5) There is a significantly higher number of $CD3^{+}$ $CD4^{+}$ T cells in synovium from Obese OA patients while there is no difference in these cells population in IPFP.
6) There is a significantly higher number of $CD3^{+}$ T cells and $CD3^{+}CD8^{+}$ T cells in synovium in comparison to IPFP from the same donor. The ratio of CD4/CD8 T cells is significantly higher in synovium from obese in comparison to lean OA patient. There is no such difference in IPFP.

# Chapter 7: The differences in obesity-related markers in osteoblasts, cellular and structural changes in subchondral bone from lean and obese OA patients

Skeletal bone is not only the biggest scaffold within the body and hematopoietic cells producer, but is also considered potent endocrine organ (Malekipour et al., 2013; Oldknow et al., 2015). Subchondral bone is a part of the knee having direct contact with cartilage. It has high porosity and is easily penetrable which allows the communication of bone and cartilage. Subchondral bone consists of a subchondral plate, in direct contact with cartilage and the subchondral trabeculae/subarticular spongiosa (Madry et al., 2010). Subchondral bone density and strength can adapt to mechanical loading. Subchondral trabecular bone is important in shock-absorbing and providing sufficient nutritional supply.

It is an active component in pathological changes during OA development (Grynpas et al., 1991; Hayami et al., 2004). Bone modelling and remodelling depend on two main cell types – osteoblasts and osteoclasts (Ducy et al., 2000; Adamopoulos et al., 2015). Osteoblasts are important in new-bone formation, while osteoclasts in bone resorption.

Osteoblasts derive from mesenchymal stem cell and their main role is in initial bone formation in both synthesis and mineralisation, in addition to bone remodelling at later stages (Ducy et al., 2000). They are also responsible for regulation of osteoclast differentiation and activity in bone resorption (Adamopoulos et al., 2015). Osteoblasts entrapped in bone matrix become osteocytes. They are terminally differentiated, do not proliferate and reside in lacunae and canaliculi.

Osteoclasts are multinucleated cells which differentiate from bone marrow- derived macrophages and are responsible for bone resorption. These cells are larger in size than osteoblasts and possess several nuclei (Jain et al., 2009). Osteoclasts have the

ability to resorb bone and can be found on the bone surface in shallow pits of trabecular bone. They produce specific proteins including TRAP (tartrate-resistant acid phosphatase) and various lysosomal enzymes and serine proteases. M-CSF (Macrophages Colony Stimulating Factor) plays a crucial role in osteoclastogenesis (Teitelbaum, 2000). Mice lacking M-CSF (op/op) exhibit osteopetrotic phenotype (Wiktor-Jedrzejczak et al., 1982). Together osteoblasts and osteoclasts maintain bone homeostasis (Adamopoulos et al., 2015).

Micro-computed tomography (µCT) is an X-ray based 3D imaging technique. It uses a standard CT (computed tomography) method with high-intensity X-rays. The CT method is based on computerised combined X-ray images taken from different angles and produces a cross-sectional image. High-intensity X-rays used in µCT method allow the use of micrometre range pixel size and 360$^{o}$ degree rotation platform to obtain a very detailed 3D image. It is a non-invasive technique that can be used to calculate following values:

Bone Volume/Total Volume (BV/TV) - which represents the volume of interest (VOI) that is occupied by mineralised bone (Bone Volume). BV/TV is normally presented as a percentage value. It is often used to evaluate changes in bone volume density during the pathogenesis of many diseases including osteoarthritis and osteoporosis (Xiao et al., 2015). Trabecular Thickness (Tb.Th.) represents an average thickness of trabeculae in the analysed volume of interest. Hildebrand and Ruegsegger et al defined local thickness as a diameter of the largest sphere which includes a certain point and can be fitted inside analysed structure (Hildebrand et al., 1997). Trabecular Spacing (Tb.Sp.) is represented by the average space between bone structures in the volume of interest. It is determined using the mean diameters of fitting maximal spheres fitted into the non-bone structure (Hildebrand et al., 1999). Trabecular Number (Tb.N.) is a number of trabeculae per unit length (1/mm) (Kim et. Al., 2015). Bone Mineral Density (BMD) is defined as the volumetric density of bone measured in g/cm$^3$ calculated with scans of a calcium hydroxyapatite phantom with known density (Miller et al., 1999).

BMD positively correlates with OA in the knee and spine (Hart et al., 1994) but there is no correlation of BMD in hand OA (Sowers et al., 1999). Studies of bone resorption markers are contradictory, some reporting increases (Stewart et al., 1999) other decreases (Sowers et al., 1999) in OA where the difference correlates with methodology: X-Ray CT or photon measurement, sample location and size.
Since it is difficult to analyse OA-affected compartments of the knee joint (such as femoral condyles and tibial plateau) due to different stages of bone sclerosis in OA patients, minimal or no OA-affected compartments of the joint called posterior lateral femoral condyles were analysed. These are also the least weight-bearing region in the knee joint. Their location is shown in Figure 7.1.

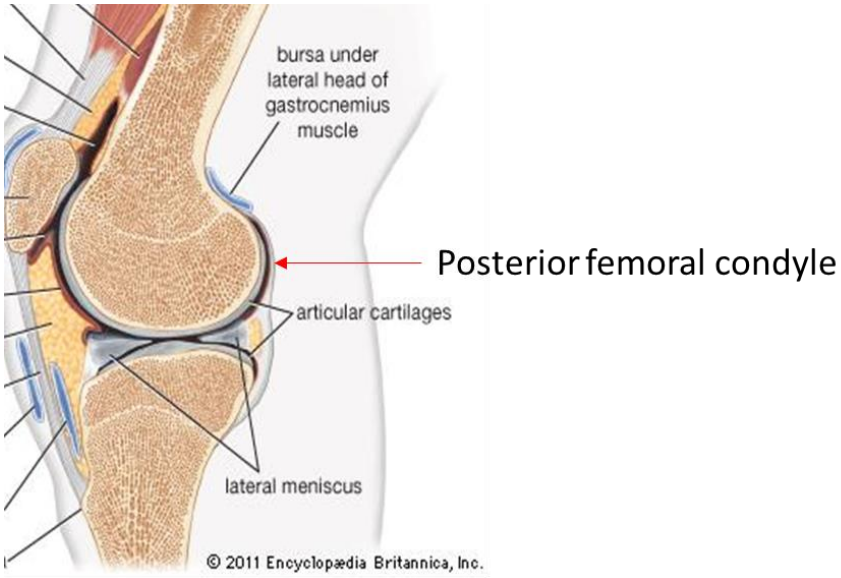



**Figure 7. 1 Knee parasagittal section-lateral to the midline of the knee with posterior femoral condyle indicated.**

Image adapted from *Encyclopedia Britannica inc*.

It is important to characterize the molecular role of cells lying in the subchondral part of the joint, and particularly their behaviour in obesity. Additional information including bone density could help with the description of changes in OA tissue during weight gain. Many other adipose-derived factors take part in bone remodelling as well. It would be interesting to connect molecular and physical changes in this part of the joint.

## 7.1 Aims

Specifically, the aims were:

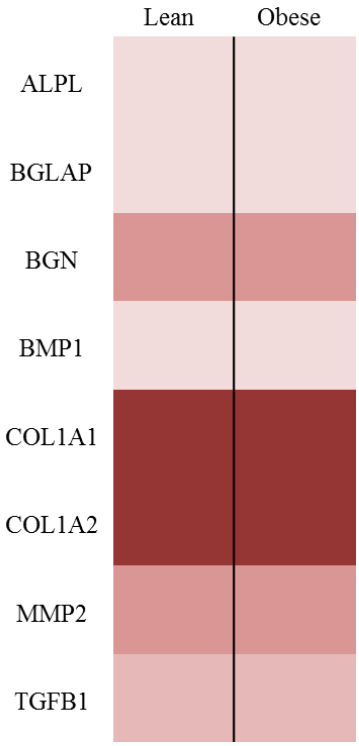
1) To investigate the obesity-related gene expression difference in osteoblasts obtained from obese and lean patients with OA.
2) To describe the changes in the bone microstructure of the minimally OA-affected and least-weight bearing compartment of the knee in obese and lean patients with OA.

## 7.2 Results

### 7.2.1 Osteoblast-specific gene expression in human primary osteoblasts from OA patients

Gene expression of osteoblast isolated from subchondral region of patellofemoral compartment from lean and obese subjects with OA has been analysed by Real Time PCR technique. Osteoblast-specific genes included: alkaline phosphatase (ALPL), osteocalcin (BGLAP), biglycan (BGN), Bone-Morphogenic Protein 1 (BMP1), collagen I (COL1A1, COL1A2), matrix metalloproteinase 2 (MMP-2) and Transforming Growth Factor β (TGFB-1) using Osteogenesis RT2 Real Time Profiler (Qiagen). The choice of osteoblast-related genes was based on supplier suggestions. Results presented in Table 5 as a heat map (with lighter colour indicating lower and darker colour indicating higher expression).

**Table 4 Human Primary Osteoblast gene expression characterisation.**




Expression of osteoblast-specific genes: ALPL, BGLAP, BGN, BMP1, COL1A1, COL1A2, MMP-2 and TGFB-1 has been confirmed using the commercially available Osteogenesis RT2 Real Time

Profiler. cDNA from 3 donors per each group (Lean and Obese) has been pulled and Real Time PCR has been conducted.

The data obtained (Table 5) confirmed that human primary osteoblasts expressed osteoblasts related genes.

### 7.2.2 Obesity-specific gene expression in human primary osteoblasts from OA patients

Obesity-related genes including ADIPOQ, ADIPOR1, ADIPOR2, LEP, LEPR, PPARγ, CCL2, NAMPT expression was investigated in osteoblasts obtained from Lean and Obese OA patients (Figure 7.2).

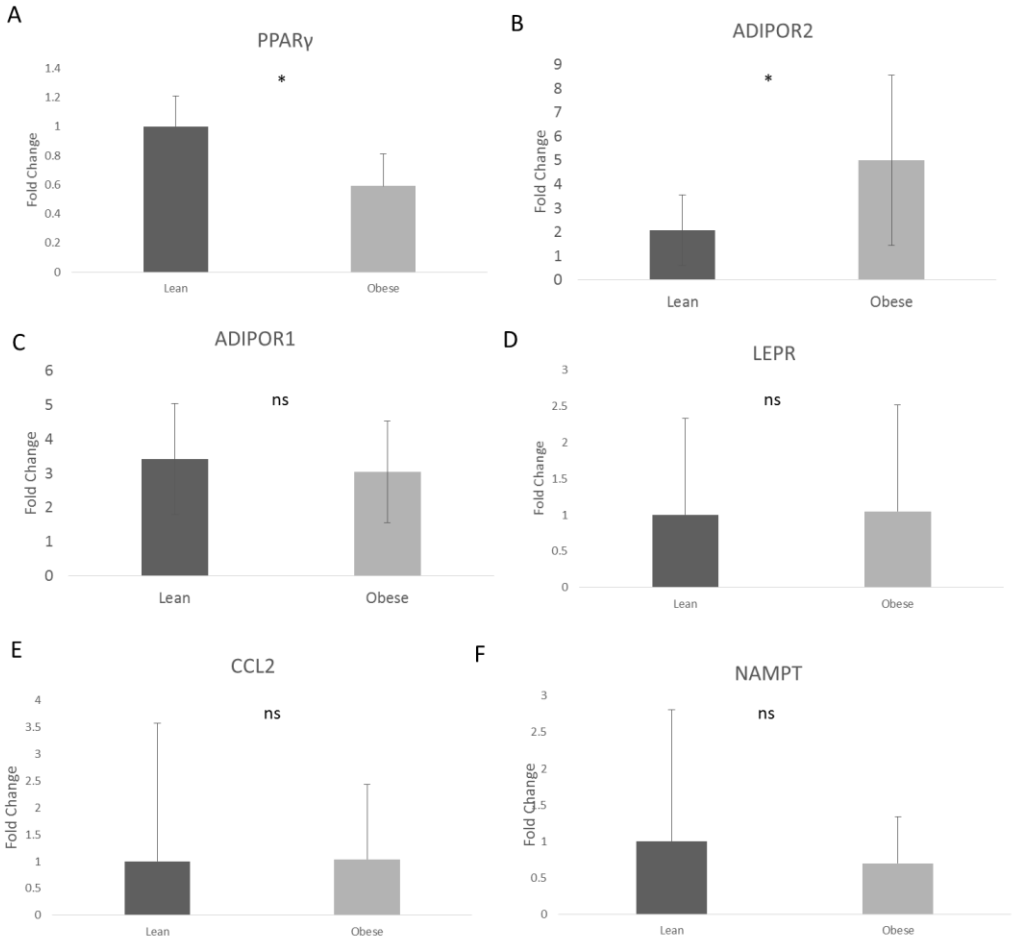



**Figure 7. 2 Quantitative PCR analysis of PPARγ (A), ADIPOR2 (B), ADIPOR1 (C), MCP-1/CCL2 (D) and NAMPT (visfatin) (E) expression in osteoblasts obtained from different OA patients (A-E).**
Two groups termed "Lean" (n=5) and "Obese" (n=7) were defined according to BMI as described before. B2M was used to normalise gene expression. The -ΔΔCT method was used to investigate fold

change in gene expression. The mean value of Lean Group was used as a calibrator. The control values were expressed as 1 to indicate a precise fold change value for each gene of interest. SEM bars are shown. p values < 0.05 were considered significant and indicated as *, NS-non-significant.

Real Time PCR revealed that both adiponectin and leptin are expressed by osteoblasts but at a very low level (data not shown). Osteoblast expression of ADIPOQ, ADIPOR1, CCL2/MCP-1, LEP, LEPR and NAMPT was not significantly different between patient groups. However, expression of adiponectin receptor ADIPOR2 was significantly higher and of PPARγ significantly lower in obese patients as compared to lean patients. Figure 7.1). Osteoblasts expressed ADIPOR1 significantly higher than ADIPOR2 (see Appendix 9).

### 7.2.3 OA Subchondral bone microstructure

The microstructure of the Femoral Condyles from OA patients was assessed by the μCT method as described in Materials and Methods (Chapter 2).

An example of Femoral Condyle analysis is presented in Figure 7.3.

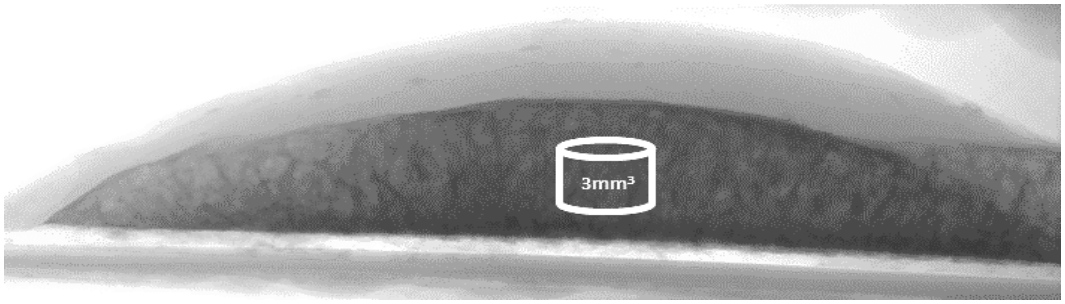



**Figure 7. 3 Example of μCT analysis of Femoral Condyle microarchitecture.**
The Volume of Interest (VOI) was defined as $3mm^3$ cube below subchondral plate region in non-weight bearing Femoral Condyle from OA patients. Patients were divided into Lean and Obese as summarised in Table4.

24 Femoral Condyles were analysed by the µCT method. Patients summary is shown in Table 6.

**Table 5 Summary of the patients µCT Femoral Condyle analysis.**

| | Mean Age | SD | P value | n |
|---|---|---|---|---|
| Lean Female | 70 | ±7 | NS | 6 |
| Obese Female | 66 | ±5 | NS | 7 |
| Lean Male | 68 | ±6 | NS | 5 |
| Obese Male | 67 | ±5 | NS | 6 |

Bone microarchitecture values (BV/TV, Tr.Th., Tr.Sp., Tr.N. and BMD) were calculated using Skyscan® software and presented in Figures 7.4-7.5.

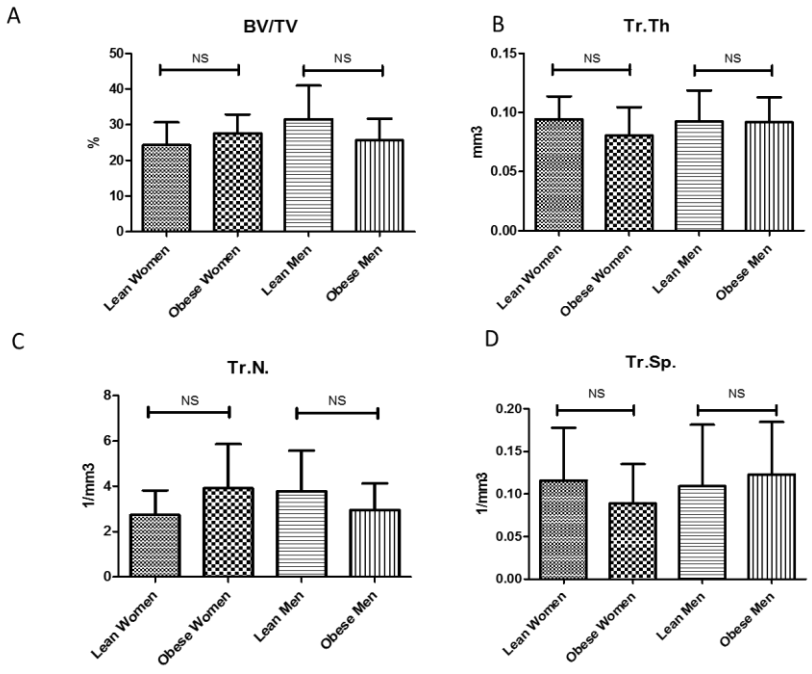



**Figure 7. 4 A) BV/TV B) Tr.Th C) Tr.N. D)Tr.Sp. values were calculated from each volume of interest taken from each patients.**
Patients were divided into Lean and Obese as well as in regards to gender. Patients summary in Table 6. Mean values of the parameters±SD per group are presented. NS-not significant.

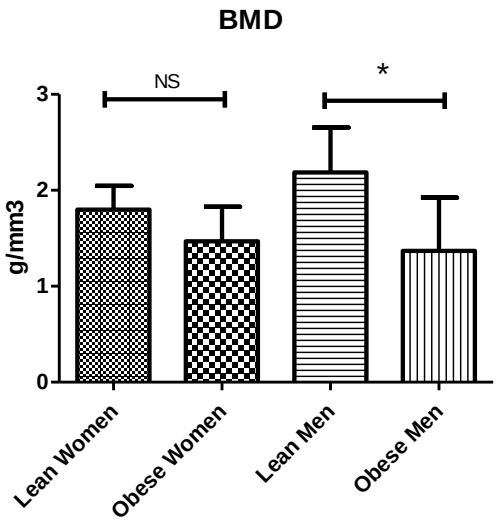



**Figure 7. 5 BMD value was calculated from each volume of interest taken from each patients.** Patients were divided into Lean and Obese as well as in regards to gender. Mean values of BMD ±SD per group are presented. p values < 0.05 were considered significant and indicated as *, NS-Not significant. BMD values were calculated by Dr Rob Wallace.

µCT data suggest that there is no difference between patient groups in any of the Bone microstructure parameters measured, including BV/TV, Tr.Th., Tr. Sep. and Tr. Nr. regardless of the gender. (Figure 7.3). However, the BMD parameter (calculated by Dr Rob Wallace) while not different between obese and lean female patients was significantly lower in obese male patients than in lean ones (Figure 7.5).

### 7.2.3.1 Subchondral bone adipose tissue macrophages in OA patients.

The presence of $CD68^+$ cells within subchondral bone marrow was investigated by immunohistochemical method (Figure 7.6).

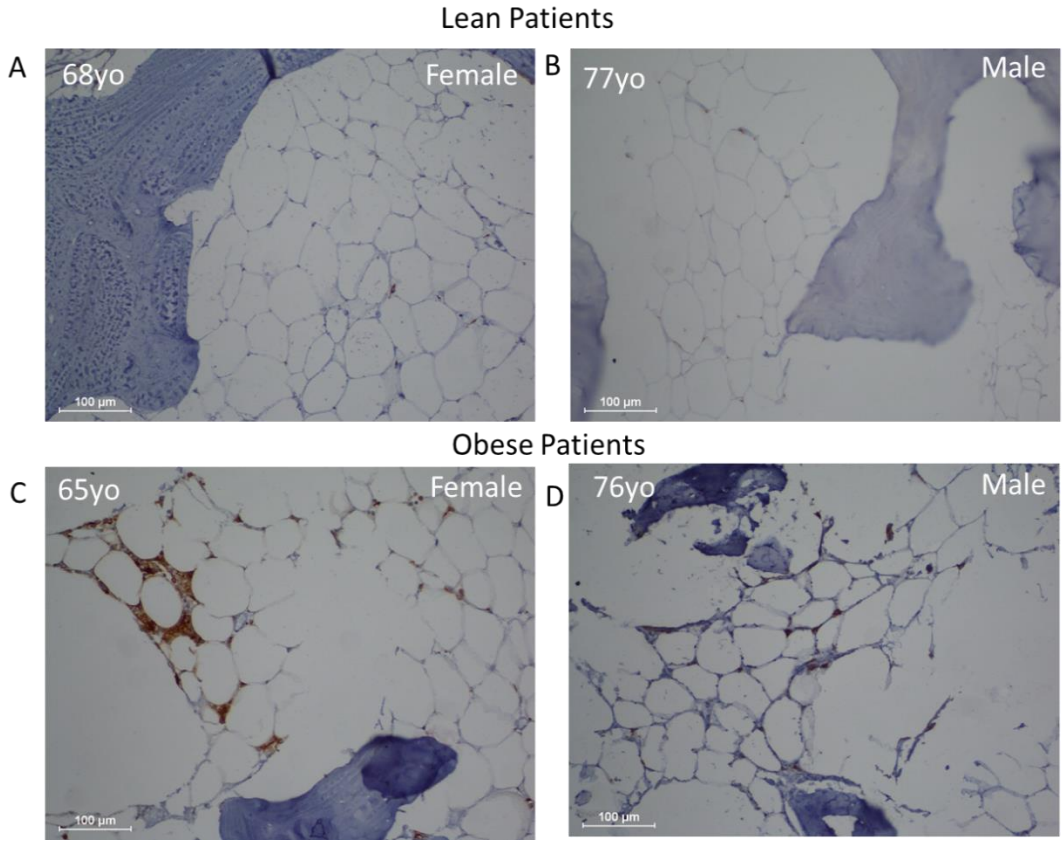



**Figure 7. 6 Subchondral bone $CD68^+$ cells in bone marrow.**
IHC staining of paraffin-embedded subchondral bone samples (n=3 for each group) confirmed the expression of $CD68^+$ positive cells (brown colour) in bone marrow adipose tissue. Different patients (A-D) are presented.

Data (Figure 7.5) suggest that there may be a higher number of $CD68^+$cells in subchondral bone adipose tissue from obese patients with OA.

## 7.3 Conclusions and Summary

In conclusion, the data obtained revealed a number of important points:

1. Osteoblasts from patients with OA express a number of obesity-related genes including ADIPOQ (adiponectin), LEP (leptin), their receptors (ADIPOR1, ADIPOR2, LEPR), NAMPT, monocyte-chemotactic protein 1 (CCL2/MCP-1) and PPARγ.
2. Osteoblasts from patients with OA express a higher level of adiponectin receptor ADIPOR1 than ADIPOR2.
3. Osteoblasts from lean and obese patients with OA differ in expression of genes connected with the pathogenesis of obesity. There is a significantly lower expression of PPARγ and higher ADIPOR2 in obese patients' osteoblasts in comparison to lean ones. No differences in other expression of investigated genes ADIPOQ, LEP, LEPR, ADIPOR1, NAMPT and CCL2/MCP-1 was found.
4. No differences in bone microstructure parameters were found between lean and obese subjects with OA. Furthermore, no difference in bone mineral density between lean and obese in women but significantly lower BMD in obese males was found in comparison to lean ones.
5. There was a higher number of CD68 positive cells in bone marrow adipose tissue in obese patients in comparison to lean ones found.

# Chapter 8: General Discussion and Future Perspectives

Mechanical loading is commonly considered to be the main contributor to the pathogenesis of OA. However, emerging data suggest that molecular factors are of high importance as well (Guilak et al., 2004). The current knowledge of proteins playing a major role in OA helps in better understanding the disease as a whole. Recent studies have reported that obesity-related molecules have a great impact on the development of OA.

Obesity is involved in dysfunction not only of adipose tissue but also in a number of other organs within the body. OA is now considered as a systemic disease, which affects all tissues within the knee joint. Knee articular tissues are potent producers of adipose tissue-associated markers. Several proteins produced by adipose tissue are considered potent players in OA progression. Data presented here and by others (Rai et al., 2014) suggest that expression and secretion of some molecules is altered in obese and lean individuals with OA.

The limitation of the study were: first of all, patients with end-stage OA were diagnosed only based on X-ray by orthopaedic surgeon. No cartilage damage scoring (such as Kellgren and Lawrence system) to classify OA stage has been used. Which may lead to differences/variation in obtained data. Secondly, this study doesn't include non-OA affected subjects. Therefore the results obtained here may be not associated with aetiology/pathogenesis of OA but simply reflecting the lean or obese status of analysed patients.
The main strength of this study was the number of analysed samples at gene expression level. Two different set of samples have been used for RT-PCR (26) and Real Time PCR (20) giving in total 46 different patients analysed.

## 8.1 Adipokines and adipose tissue-related markers

### 8.1.1 Adiponectin

Adiponectin is considered to be a protective adipokine in most obesity-related pathologies. It increases insulin sensitivity, reduces circulating fatty acids and triglyceride deposition in liver and muscle (Fantuzzi, 2005). It is known to attenuate TNF-α pro-inflammatory action in endothelial cells and thus to reduce the progression of atherosclerosis (Ouchi et al., 1999). Adiponectin also protects against cardiac inflammation mediated by angiotensin II by increasing the autophagy of macrophages. Therefore, adiponectin is considered to be an anti-inflammatory protein in terms of T2DM and atherosclerosis.

However, the role of adiponectin in OA is still controversial. The majority of authors, state it has a pro-inflammatory role in cartilage metabolism (Chen et al., 2006; Kang et al., 2010). Nevertheless, circulating levels of adiponectin have been reported to be higher than that of synovial fluid level in some individuals (Presle et al., 2006; Honsawek et al., 2010; Gandhi et al., 2011). Furthermore, serum adiponectin level has been found to correlate negatively with the BMI of OA patients (Chen et al., 2006; de Boer et al., 2012) and its synovial fluid level to correlate negatively with degradation markers including Aggrecan I and Aggrecan II. Thus, adiponectin may also be postulated as an anabolic factor.

Hence, one of the goals of the current studies was to investigate the difference in adiponectin production by the local tissues within the knee of lean and obese OA patients. Table 7 summarises the obtained data in regards to adiponectin and Adiponectin Receptor (ADIPOR1 and ADIPOR2) expression in analysed tissues.

**Table 6 Summary of adiponectin and its receptors expression data**

| Marker | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| Adiponectin | ↔ | ↓ | ↔ | ↔ |
| ADIPOR1 | ↑ | ✖ | ↔ | ↔ |
| ADIPOR2 | ↔ | ✖ | ↔ | ↑ |

**Legend**

↑ Expressed higher ↓ Expressed lower ↔ Expressed at the same level ✖ Not Expressed

Adiponectin expression was detected in monolayer chondrocyte cell culture on a very low level. Francin et al., studied adiponectin expression in different chondrocytes cell culture models and found that adiponectin was present in cartilage but its expression is down-regulated in monolayer cell culture (Francin et al., 2011). There are discrepancies regarding adiponectin receptors expression in cartilage. Here chondrocytes from OA patients expressed both adiponectin receptors with significantly higher expression of ADIPOR1 compared to ADIPOR2. Further, this difference was significantly higher in chondrocytes from obese as compared to lean patients, which may imply an important role for this receptor in the OA process in obese patients. Other authors have recently reported that these two receptors are down-regulated in osteoarthritic compared to healthy chondrocytes (Wang et al., 2014). Some authors report that only ADIPOR1 is present in cartilage (Chen et al., 2006), while others indicate that both receptors are present, but with different localisation within the cartilage (Kang et al., 2010). It has been suggested that binding of adiponectin to ADIPOR1 receptor increases MMP-3 expression in chondrocytes (Tong et al., 2011). Elevated expression of this receptor could suggest that adiponectin has an important function in OA chondrocytes.

The present study also identified adiponectin expression in OA Synovium (Chapter 4) and IPFP (Chapter 5) which concurs with previous findings (Presle et al., 2006; Tan et al., 2009). In addition, the presented data have demonstrated that adiponectin had a significantly lower expression both at gene and protein level in the synovium from obese subjects with OA but remained at the same level in their IPFP. Adiponectin was also secreted at a lower level in the media of synovium explants from obese patients but not from their IPFP.

Although some researchers consider adiponectin to be a pro-catabolic protein in the pathogenesis of OA (Xie et al., 2007; Gomez et al., 2011; Tong et al., 2011) there is also evidence that it has a pro-anabolic protective role in cartilage (Bastiaansen-Jenniskens et al., 2012). Obtained data here (Figure 4.8, 5.7) suggest that both synovium and IPFP were capable of producing adiponectin in the 'ng/ml' range. Tan et al. and others (Koch et al., 1992; Presle et al., 2006; Tan et al., 2009; Gandhi et al., 2010) have reported that the synovial fluid adiponectin level reached 400-600ng/ml in OA while its serum level went as high as 10 µg/ml. It was interesting to note that the majority of studies, which have investigated adiponectin's "pro-inflammatory/catabolic role" in OA, have used superoptimal doses of that protein (Conde et al., 2012; Lee et al., 2012) as high as 100 µg/ml (Ehling et al., 2006).

In paired samples (of synovium and IPFP) from lean subjects with OA adiponectin levels (both mRNA and protein) remained at the same level between these two tissue depots. In contrast, in Obese subjects, IPFP produced the significantly higher level of adiponectin than synovium. These differences could be explained by the developmental origin of those two tissues. Subcutaneous Adipose Tissue (SAT) was shown to produce more adiponectin than Visceral Adipose Tissue (VAT) during weight gain (Lihn et al., 2004; Hernandez-Morante et al., 2007). In addition, the IPFP was shown to produce more adiponectin than paired subcutaneous adipose (SAT) tissue from the same donor (Distel et al., 2009; Klein-Wieringa et al., 2011) which may suggest IPFP to be a potent adiponectin producing fatty depot. In the future, it would be very interesting to analyse the origin of two adipose tissue depots (synovial and IPFP) within the knee.

Osteoblast expression of adiponectin and its receptors has already been confirmed by others (Berner et al., 2004). Presented data also show that primary human osteoblasts expressed the adiponectin gene, although at a very low level. In this study results show that similar to chondrocytes, osteoblasts expressed significantly higher ADIPOR1 than ADIPOR2 (Appendix 9). ADIPOR1 is more ubiquitously expressed than ADIPOR2 in the majority of the tissues except liver (Yamauchi et al., 2003) and has been shown to be expressed at a higher level than ADIPOR2 in osteosarcoma cell line Saos-2 (Pacheco-Pantoja et al., 2014). The data presented here didn't show a significant difference in adiponectin and ADIPOR1 gene expression between lean and obese patients' osteoblasts. However, ADIPOR2 gene expression was significantly higher in osteoblasts from obese OA patients in comparison to lean ones (Figure 7.2) , whereas chondrocytes from obese patients, expressed significantly higher ADIPOR1 (Figure 3.3 and 3.7). It may suggest that adiponectin plays a different role in these two tissue types in obesity.

ADIPOR1 and ADIPOR2 are transmembrane receptors sharing about 67% homology. They both are important in adiponectin-mediated glucose level regulation and fatty acid oxidation. ADIPOR1 is involved in activation of 5' adenosine monophosphate-activated protein kinase (AMPK) pathway while ADIPOR2 induce PPARα receptor activation cascade.

Adiponectin knockout mice have impaired bone growth and adiponectin inhibits RANKL expression (an important factor in osteoclast activity), in macrophage cell-line (Tu et al., 2011). Conversely, other authors suggest that adiponectin stimulates RANKL and down-regulate OPG expression in osteoblasts (Luo et al., 2006; Pacheco-Pantoja et al., 2014) by ADIPOR1 mediated pathway. This could postulate that this protein has a dual role in bone homeostasis and be responsible for balancing the bone remodelling processes. There is no available literature about the precise role of ADIPOR2 in osteoblasts.

Adiponectin has been also shown to upregulate MMP-3 (important cartilage destruction/pro-catabolic enzyme) in cartilage through ADIPOR1 but not ADIPOR2

(Tong et al., 2011). What's more, adiponectin-induced migration of chondrosarcoma cells through the AMPK mediated pathway (Chiu et al., 2009).

It is interesting how a small number of researchers focus on adiponectin receptor in bone and cartilage as well as on the full mechanism of adiponectin action in those tissues. It would be interesting to further explore the role of this protein and its receptors in knee joint tissues by small molecules targeting or knockout experiments.

### 8.1.2 PPARγ

PPARγ is a nuclear receptor in the family of receptors, which is important in the regulation of many processes within the cells, including (1) glucose and lipid homeostasis, (2) adipogenesis, (3) the immune response and (4) cell differentiation. PPARγ expression in obesity is still controversial: it has been reported to be both up- and down-regulated in obese tissues (Vidal-Puig et al., 1997; Hammes et al., 2012).

Several studies have described the expression of PPARγ in human cartilage and chondrocytes (Francois et al., 2004; Shao et al., 2005; Grogan et al., 2013). The putative protective role of PPARγ against OA is supported by reports that PPARγ expression is higher in normal as compared to OA cartilage (Afif et al., 2007) and that mice with PPARγ knocked out develop OA (Vasheghani et al., 2015). Many PPARγ agonists (called thiazolidinediones/TZD) are now used as potent anti-diabetic and anti-inflammatory factors (Monsalve et al., 2013). It has been suggested that PPARγ agonists may also be potential therapeutic drugs for arthritic diseases including RA (Giaginis et al., 2009). In addition, the PPARγ agonist – pioglitazone, has been shown to inhibit OA progression in a guinea pig experimental model of OA (Kobayashi et al., 2005). Pro-inflammatory cytokines such as IL-1β and TNF-α have been reported to down-regulate PPARγ expression in human chondrocytes (Afif et al., 2007) thereby negating the endogenous beneficial effect of PPARγ.

Data obtained in this study are summarized in Table 8.

**Table 7 Summary of PPARγ expression data**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| PPARγ | ↓ | ↓ | ↓ | ↓ |

**Legend**
↓ Expressed lower

In the presented chondrocyte monolayer model, it was possible to demonstrate lower expression of both PPARγ gene and protein expression in obese patients cells. Further studies (including mass spectrometry) would help to investigate *in vivo* difference in PPARγ cartilage expression. Demonstrating a key role for reduced PPARγ activity in the pathogenesis of OA in obese patients is challenging as both mRNA and protein isolation from cartilage are very difficult. It was not possible to show differences in PPARγ expression in obese vs. lean patients cartilage

PPARγ has been described in RA synovium mainly in macrophages and synovial fibroblasts in the lining area, but also in fibroblasts and endothelial cells in sublining compartments (Kawahito et al., 2000). Immunohistochemical staining in this study confirms expression of PPARγ in several cell types in OA Synovium and IPFP. Furthermore, obtained data suggest that PPARγ gene and protein is expressed at lower level in both of those tissues in obesity (Chapter 4 and 5). In contrast, it has been reported that PPARγ expression was up-regulated in the IPFP at the endstage of OA (Clockaerts et al., 2012) however, no distinction between obese and lean patients was made. PPARγ anti-inflammatory role was confirmed by Mouilin et al. and others, who described that PPARγ agonists inhibited IL-1β mediated cyclooxygenase 2 (COX-2) production in synovial fibroblasts (Simonin et al., 2002; Moulin et al., 2006).

Comparison of PPARγ expression between paired IPFP and synovium showed it is significantly higher in IPFP. Some studies have demonstrated that PPARγ is expressed at a lower level in visceral than subcutaneous adipose tissue (Hammes et al., 2012; Garin-Shkolnik et al., 2014). Other studies have shown a significantly lower expression of PPARγ in the IPFP in comparison to paired subcutaneous adipose tissue (Distel et al., 2009). Therefore, this significant difference between IPFP and synovium in PPARγ expression can suggest a potential different developmental origin as was seen in adiponectin expression.

Furthermore, PPARγ is a known activator of adipogenesis and a repressor of osteoblastogenesis. PPARγ agonists including rosiglitazone or pioglitazone (both from TZD family) are known to cause bone resorption in mice (Ali et al., 2005). However, pioglitazone has been postulated to increase bone mineral density in induced arthritis (Koufany et al., 2013). PPARγ depletion in bone marrow-derived mesenchymal stem cells facilitated pro-osteoblastogenic phenotype, with increased bone formation markers and trabecular architecture (James et al., 2014). PPARγ is also a confirmed promotor of osteoclastogenesis and positive modulator of bone resorption. PPARγ deletion in hematopoietic but not mesenchymal cells resulted in osteopetrosis. In addition, PPARγ ligand stimulation accelerated osteoclast maturation and bone resorption (Wan et al., 2007). Osteoclasts in mice display M2 phenotype and PPARγ agonist stimulation increase expression of M2 type markers including CD206 expression.

The data presented show that primary human osteoblasts express PPARγ and that its expression is significantly lower in obese patients' osteoblasts in comparison to lean ones as was seen in chondrocytes.To mimic juvenile obesity Shu et al. fed young mice with a high-fat diet and observed lowered bone quality, increased osteoclast activity which correlated with increased total bone PPARγ expression. Interestingly the group also found increased osteoblast activity in the same model (Shu et al., 2015). Similarly to adiponectin the PPARγ role in bone may be to maintain bone homeostasis.

To sum up, PPARγ expression was lower in all obese tissue/cell types investigated in the present study. These results suggest that PPARγ could be a potent OA/obesity target in future research, especially given that PPARγ agonists are already used in treating obesity-related diseases for example T2DM.

### 8.1.3 Visfatin

Visfatin was initially described as an adipokine produced predominantly by visceral fat (Fukuhara et al., 2005), however, it has now been described in many cell and tissue types. It has three different roles which are reflected by its three different names: (1) PBEF (from Pre-B-cell colony-Enhancing Factor 1) reflecting its role as an active cytokine involved in lymphocyte survival, (2) NAMPT (from NictotinAMide PhosphoribosyloTransferase) reflecting its role as an intracellular rate limiting enzyme and (3) visfatin reflecting its role as a hormone involved in obesity. Nonetheless, its specific role in the pathogenesis of obesity and OA has still not been fully determined.

Visfatin has been suggested to play a pro-inflammatory role in cartilage(Gosset et al., 2008; Laiguillon et al., 2014). Furthermore, its level is elevated in the Synovial Fluid from OA patients compared to normal subjects (Duan et al., 2012) and varies between 10-50 ng/ml (Nowell et al., 2006; Rho et al., 2009). However, most of the studies postulating its pro-inflammatory role in OA have used non-physiological concentrations in *in vitro* studies (100ng/ml up to 5 μg/ml) (McNulty et al., 2011; Yammani et al., 2012). Additionally, in the majority of studies related to arthritic disease visfatin is considered only as a secretory protein with no acknowledgment of its important intracellular enzymatic role. However, this protein is very important in several intracellular pathways including the biosynthesis of nicotinamide adenine dinucleotide (NAD) a coenzyme crucial in many cellular metabolic processes.

Data from this study are summarized in Table 9.

**Table 8 Summary of visfatin/NAMPT expression**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| Visfatin (NAMPT) | ↓ | ↓ | ↓ | ↔ |

Legend

↓ Expressed lower ↔ Expressed at the same level

The results reported here confirm abundant expression of visfatin in chondrocytes both in monolayer cell culture and in cartilage. IF stainings show both cytoplasmic and nuclear location of this protein which may suggest that visfatin has not only a role as a secreted adipokine but also an intracellular enzyme in chondrocytes. Previous studies have shown that visfatin localisation within the cell is strictly correlated with cell cycle (Kitani et al., 2003) and the presence of pro-inflammatory factors including IL-1β (Romacho et al., 2013). IL-1β has also been shown to markedly up-regulate visfatin gene expression in chondrocytes (Gosset et al., 2008).

Surprisingly, in the current studies, mRNA expression of the visfatin gene (NAMPT), which has previously been reported as a pro-inflammatory adipokine in chondrocyte biology, was significantly lower in the chondrocytes from obese OA patients. Lower expression of visfatin gene and protein in chondrocytes from obese patients may suggest different molecular processes occurring in ‘obese’ chondrocytes.

Visfatin expression has been previously described in the synovium of RA and OA patients (Brentano et al., 2007; Laiguillon et al., 2014). Furthermore, visfatin secretion by IPFP has also been reported previously (Chen et al., 2010). Interestingly, visfatin is produced by the IPFP at a higher level than by paired subcutaneous fat depots mainly by cells other than adipocytes (Barth et al., 2010;

Klein-Wieringa et al., 2011). However, Chen et al reported that the synovium and IPFP produced visfatin at similar levels (Chen et al., 2010).

The data reported here also indicated that visfatin was present in OA synovium and IPFP which concurred with Chen's group's data. visfatin was expressed at similar levels in the IPFP and synovium. However, the data reported here indicated that intracellular visfatin was down-regulated at both gene and protein level in the synovium and IPFP from obese patients with OA.

Surprisingly, in this study, two bands were detected in Western Blot analysis for visfatin, 54 and approximately 72kDa, in both synovium and IPFP. The majority of published studies presented only a 54kDa visfatin band (Brentano et al., 2007). However, some investigators detected the higher molecular weight band (Laiguillon et al., 2014) but didn't discuss its presence. The 72kDA band could be a non-specific binding of the primary antibody or as yet unexplained post-translational modification of this protein. UniProt analysis of visfatin protein reveals that it has several possible ubiquitination regions (NX_P43490). Furthermore, auto-phosphorylation of the active enzyme has been also postulated (Wang et al., 2006). In the future immunoprecipitation or mass spectrometry analysis of visfatin production could help with understanding its role in OA tissues.

The NAMPT gene was expressed by the human osteoblasts analysed in this study. There was no significant difference in NAMPT expression between lean and obese patients' osteoblasts. visfatin has been reported to promote osteoblast proliferation and collagen secretion (Xie et al., 2007) and to block osteoclast differentiation (Moschen et al., 2010). Conversely, Moschen et al. in the same study showed that visfatin circulating level negatively correlated with BMD in inflammatory bowel disease.

In summary, data presented here unexpectedly show that visfatin expression is lower in chondrocytes, synovium and IPFP of obese patients than in lean ones. Further investigation of the role of intracellular/enzymatic visfatin may be helpful in explaining the role of this protein in osteoarthritic tissues.

Adipokines and obesity-related proteins combined summary is shown in Table 9.

**Table 9 Summary of Adipokines/Obesity-related proteins data.**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| Adiponectin | ↔ | ↓ | ↔ | ↔ |
| ADIPOR1 | ↑ | ✖ | ↔ | ↔ |
| ADIPOR2 | ↔ | ✖ | ↔ | ↑ |
| PPARγ | ↓ | ↓ | ↓ | ↓ |
| NAMPT(Visfatin) | ↓ | ↓ | ↓ | ↔ |

**Legend**

↑ Expressed higher ↓ Expressed lower ↔ Expressed at the same level ✖ Not Expressed

To sum up, all obtained data may suggest that there are diverse molecular patterns in the pathogenesis or progression of OA between lean and obese subjects. Differential expression of ADIPOR1 and ADIPOR2 between two cell types (chondrocytes and osteoblasts) and altered adiponectin expression in synovium and IPFP may point this protein as an important factor at the crossroad between obesity and OA. It can also suggest that all tissues forming the knee joint can have differential roles in OA pathogenesis. Furthermore, PPARγ expression was significantly lower in all obtained tissue types in obesity which can suggest an important role of this nuclear factor. Similarly, visfatin's lower expression in obese patients chondrocytes, synovium and IPFP raise importance for the further analysis of this molecule.

Although presented data don't fully explain the differences in obese and lean OA patients, they can suggest some molecular variances between those two groups. Interestingly PPARγ agonists are known inducers of adiponectin (Maeda et al., 2001;

Kudoh et al., 2011) and adiponectin receptors (Sun et al., 2006) which could partially connect data obtained in synovium but doesn't explain high adiponectin production by IPFP. Furthermore, Rosiglitazone – one of the PPARγ agonist significantly induced both visfatin and adiponectin expression in rat adipose tissue (Choi et al., 2005). Parallel down-regulation of both ADIPOQ and PPARγ has also been reported in Visceral Adipose Tissue of extremely obese subjects (Hammes et al., 2012).

## 8.2 Immune System Related Markers

### 8.2.1 CCL2

Monocyte chemotactic protein-1 (MCP-1, also known as Chemokine (C-C) ligand 2 – CCL2) is a 13.8kDa peptide with four highly conserved residual cysteines, which have chemotactic properties. MCP-1/CCL2 is an important chemokine, which stimulates macrophage chemotaxis. Its expression is upregulated in obesity (Huber et al., 2008) and has an effect on tissue pathologies including plaque formation in coronary diseases (Gonzalez-Quesada et al., 2009). It is also chemotactic for T-cells and NK cells. MCP-1/CCL-2 mediates its effect mainly by engagement of the CCR2 receptor (C-C motif chemokine receptor 2).

CCL2/MCP-1 is a major factor in the pathogenesis of articular diseases including RA (Pavkova Goldbergova et al., 2012). Its expression was confirmed in OA chondrocytes by Yuan et al., and its up-regulation in chondrocytes was detected after IL-1β and TNF-α stimulation (Yuan et al., 2001).

The data obtained in this study are presented in Table 11.

**Table 10 Summary of CCL2/MCP-1 expression data**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| CCL2/MCP-1 | ↑ | ↑ | ↔ | ↔ |

**Legend**
↑ Expressed higher ↔ Expressed at the same level

The present data show higher MCP-1/CCL2 gene expression in chondrocyte cell culture and higher protein production by cartilage from obese patients, which may suggest that ‘obese’ chondrocytes possess a more pro-inflammatory phenotype.

MCP-1/CCL2 is also produced by both synovium and infrapatellar fat pad explants as previously reported (Clockaerts et al., 2012). The data obtained confirm both mRNA and protein expression of MCP-1/CCL2 along with its secretion in synovium. MCP-1/CCL2 has been reported to be produced by fibroblast-like synoviocytes (FLS) after stimulation by pro-inflammatory cytokines (Ogura et al., 2010). Furthermore, its receptor (CCR2) can be found on fibroblast-like cells and it has been postulated to have a role in cell migration in RA (Garcia-Vicuna et al., 2004). Villiger et al showed that MCP-1/CCL2 was present in the synovial membrane of the synovium and in the perivascular area (Villiger et al., 1992). Another group (Vangsness et al., 2011) showed that the synovial fluid concentration of MCP-1/CCL2 was greater in more advanced OA. Unfortunately, the correlation with BMI was not explored in those studies. MCP-1/CCL-2 has also been reported to be expressed by synovial fluid macrophages in RA and in OA (Koch et al., 1992). Its role in RA and inflammation of synovium has been well described (Patterson et al., 2002). In samples from OA patients, MCP-1/CCL2 has previously been reported to be expressed in the sublining layer of the synovium along with the perivascular and interstitial areas of the synovium (Villiger et al., 1992).

Local production of MCP-1/CCL2 in OA was measured using both PCR and ELISA techniques. The data obtained suggested that there was higher MCP-1/CCL2 gene expression in synovium from obese patients. The MCP-1/CCL2 secretion by synovial explants was not significantly higher; however, there was a trend towards higher MCP-1/CCL2 production in the specimens of synovium from obese in comparison to lean OA patients. MCP-1/CCL2 gene was also expressed by osteoblasts, but there was no significant difference between lean and obese patients' osteoblasts.

In conclusion, obtained data may suggest that MCP-1/CCL2 can be another potential protein for further investigation in the correlation between obesity and OA.

### 8.2.2 TLR4

The innate immune system has developed several mechanisms to fight infection. Pattern-Recognition Receptors (PRR) evolved to recognise conserved structures called Pathogen-Associated Molecular Patterns (PAMPs) including Lipopolysaccharides (LPS), Mannose Binding Lectin (MBL), C-reactive protein (CRP), DNA or free fatty acids (FFA). The TLR (Toll-like receptor) family is a highly conserved group of type I transmembrane receptors which belong to PRR. TLRs play an essential role in pathogen recognition and in the innate immune response. There are more than 10 TLRs described in humans (Jialal et al., 2014). Furthermore, TLRs are also potent receptors for by-products produced by the host cells called damage-associated molecular patterns (DAMPs) including nucleic acids, low-density lipoproteins, extracellular matrix or free fatty acids, which can trigger the immune system without the actual presence of a pathogen.

The two best-characterised TLRs are TLR2 and TLR4. Both have been described as playing an important role not only in bacterial infection but also in the development of such diseases as T2DM, atherosclerosis and obesity (Reyna et al., 2008). Obesity is associated with elevated levels of circulating free fatty acids (FFA) especially saturated fatty acids (SFA). SFA are potent agonists of TLR2 and TLR4 and can induce pro-inflammatory proteins production. In obese mouse models including *ob/ob*, *db/db* and diet-induced obesity (DIO), the TLR4 expression is elevated in adipocytes (Song et al., 2006; Tsukumo et al., 2007). Whereas, TLR4 knock out mice are protected from insulin resistance and do not display elevated pro-inflammatory proteins including IL-6, TNF-α and MCP-1/CCL2 despite increased adipose tissue mass (Shi et al., 2006; Davis et al., 2008). TLR4 is a protein, which plays a fundamental role in pathogen recognition and being a very potent signal transducer in many diseases not related to bacterial infection. It can bind and respond to many DAMPs. A study conducted by Sohn et al. (Sohn et al., 2012) suggested that circulating cytokines, which could be detected in synovial fluid of OA patients, could trigger an inflammatory process in joints by binding to TLR4.

TLR4 is present in healthy cartilage (Bobacz et al., 2007) and is induced in injured cartilage (Kim et al., 2006). Bobacz et al, state that TLR4 activation induces IL-1β and down-regulates collagen II and aggrecan expression. The data obtained in the present study are presented in Table 12.

**Table 11 Summary of TLR4 expression data**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| TLR4 | ↔ | ↑ | ↔ | NA |

**Legend**

↑ Expressed higher ↔ Expressed at the same level **NA** – not assessed

Data presented here did not show any difference in TLR4 gene expression in chondrocytes and IPFP between the lean and obese OA groups. However, Synovial gene and protein expression revealed significantly higher TLR4 expression in obese samples which might suggest a more pro-inflammatory status of the synovium from obese patients with OA.

TLR4 plays a crucial role in monocytosis and the accumulation of macrophages in adipose tissue of obese patients (Nagareddy et al., 2014). Macrophage cell death, which occurs in the lipotoxic condition of obesity is mediated by TLR4 (Schilling et al., 2013).

In humans, VAT express higher levels of TLR4 than SAT. Furthermore, TLR4 expression is up-regulated in obese human VAT but not SAT (Catalan et al., 2012). TLR4 deficiency promotes an alternative (M2) activation of macrophages (Orr et al., 2012). In addition, TLR4 and PPARγ play important roles in the regulation of chemokine expression in adipocytes and macrophages (Nguyen et al., 2012).

In summary, data presented here suggests that TLR4 is expressed at a significantly higher level in the synovium of obese compared to lean OA patients which indicate

that TLR4 may have an important role in linking OA and obesity. Additionally, chondrocytes and IPFP TLR4 expression is not different between two analysed groups. TLR4 expression was not analysed in osteoblast and can be a matter of future investigation.

### 8.2.3 Vascular Cell Adhesion Molecule 1 (VCAM-1)

Vascular Cell Adhesion Molecule 1 (VCAM-1) is an integrin which is important in the migration process of immune cells through vessels. It binds to the Very Late antigen 4 (VLA-4) integrin, which is expressed by many immune cells including T-cells and monocytes. VCAM-1 mRNA and protein expression has been reported to be up-regulated in obese VAT (Bosanska et al., 2010). VCAM-1 has also been reported to be expressed in human synovial tissue, but only in the FLS and not in the fatty layer. Fibroblast-Like Synoviocytes are able to express VCAM-1 on their surface and its expression is induced by pro-inflammatory cytokines including IL-1β (Morales-Ducret et al., 1992). There is an increased serum level of soluble VCAM-1 in erosive hand OA (Pulsatelli et al., 2013) and this protein is suggested to be a potent predictor of hand (Kalichman et al., 2011), hip and knee (Schett et al., 2009) OA. Up-regulation of VCAM-1 expression by synovial cells is suggested to enhance leukocyte extravasation and adhesion of such cells as monocytes (Shang et al., 1998) and B-lymphocyte (Burger et al., 2001). VCAM-1 mediates adhesion of cells (lymphocytes, monocytes or eosinophils) to blood vessel walls for migration into tissues in inflammatory responses. It is expressed in normal chondrocytes and its expression is regulated by cytokines such as IL-1β or TNF-α (Kienzle et al., 1998). VCAM-1 expression is also induced by leptin and adiponectin (Conde et al., 2012).

Obtained data are summarized in Table 13.

**Table 12 Summary of VCAM-1 expression analysis**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| VCAM-1 | ↔ | ↑ | ↔ | NA |

**Legend**
↑ Expressed higher ↔ Expressed at the same level **NA** – not assessed

The data presented here may suggest that there was no significant difference in chondrocytes and IPFP VCAM1 expression, but that was a higher VCAM1 mRNA production in obese OA synovium. Flow Cytometry analysis also suggested that the VCAM-1 protein expression in a stromovascular fraction of synovium was higher in obese subjects compared to lean ones. These data can possibly suggest VCAM-1 is an important molecule in the more pro-inflammatory phenotype of OA in obese patients. Increased Synovial VCAM-1 expression may suggest an increased immune cell intravasation in obese in comparison to lean OA patients. VCAM-1 expression was not analysed in osteoblasts and is a matter for future investigation.

**Table 13 Immune Cells related protein expression summary**

| Expression Obese (in comparison to Lean) | Chondrocytes/ Cartilage | Synovium | IPFP | Osteoblasts/Bone |
|---|---|---|---|---|
| CCL2/MCP-1 | ↑ | ↑ | ↔ | ↔ |
| TLR4 | ↔ | ↑ | ↔ | NA |
| VCAM1 | ↔ | ↑ | ↔ | NA |

**Legend**
↑ Expressed higher ↔ Expressed at the same level **NA** – not assessed

Macrophage-related proteins including MCP-1/CCL2 and TLR4 in synovium of Obese subjects may suggest an augmented influx of those cells and maybe more pro-inflammatory status of this tissue. Higher VCAM1 expression in synovium of Obese OA patients may suggest increased signal in this tissue for immune cells intravasation.

### 8.2.4 Immune cells content in OA fatty tissues samples

Macrophages are the most abundant leukocytes in adipose tissue. In lean patients, the majority of them display anti-inflammatory M2 phenotype (Lumeng et al., 2007) and express anti-inflammatory cytokines including IL-10 which helps in maintaining insulin sensitivity. In obese subjects, the number of macrophages increases and there is a "switch" from M2 type to M1 type and production of pro-inflammatory proteins including IL-1β, TNF-α, IL-6. Pro-inflammatory macrophages aggregate around dying and necrotic adipocytes, in addition to contributing to further adipose tissue inflammation. With weight gain up to 30% of genes upregulated in obesity are related to macrophages (Weisberg et al., 2003). However, the macrophage content in adipose tissue differs between fat depots.

Obtained data in Chapter 6 are summarized in Table 15

**Table 14 Summary of macrophage-related markers in SVF fraction of synovium and IPFP analysis**

| Immune Cell Content Obese (in comparison to Lean) | Synovium | IPFP |
|---|---|---|
| CD14CD45 | ↑ | ↑ |
| CD14CD206 | ↑ | ↑ |
| CD14CD86 | ↑ | ↔ |
| CD36 | ↑ | ↔ |
| HLA-DR | ↑ | ↑ |

**Legend**
↑ Expressed higher ↔ Expressed at the same level

The data obtained in this study suggested that the population of $CD45^{+}CD14^{+}$ cells was higher in the synovium and IPFP from obese OA subjects in comparison to lean ones. The number of $CD14^{+}CD206^{+}$ cells was also significantly higher in the synovium and IPFP from obese patients. CD206 is considered to be an M2-type macrophage marker in mice and has been reported to be up-regulated in humans with obesity (Bourlier et al., 2008; Wentworth et al., 2010; Haase et al., 2014). CD206 is a mannose receptor C type 1 (MRC1) and belongs to the mannose receptor family. It can be found in numerous cell types including macrophages, immature dendritic cells, epithelium and smooth muscle cells. It recognises terminal mannose and other monosaccharides which can be found on the surface of many microbial glycoproteins. Its main function is phagocytosis and internalisation of pathogens.

CD206 plays an important role in the resolution of inflammation by M2 macrophages as it recognises glycoproteins (including the lysosomal hydrolases), which play a role in pathogen elimination but with prolonged exposure are potentially harmful to the host.

CD86 is an M1 macrophage marker that has been reported to play an important role during foam cells formation in atherosclerotic plaques (Chavez-Sanchez et al., 2014). CD86 has been reported to be present in both IPFP and the synovium (Bastiaansen-Jenniskens et al., 2012; Fahy et al., 2014). Furthermore, the medium from synovial M1 type macrophages, which express CD86 is able to down-regulate chondrogenic gene expression during mesenchymal stem cells chondrogenic differentiation (Fahy et al., 2014). Results presented here indicate that the number of $CD86^{+}CD14^{+}$ positive cells was significantly higher in the synovium from obese patients but there was no difference in these cells number between obese and lean patients in the samples of the IPFP.

CD36 is a transmembrane glycoprotein, which is a member of the scavenger receptor family. It is expressed on various types of cells including monocytes and macrophages, platelet and myocytes. It has a number of ligands including oxLDL and long chain fatty acids. CD36 is also a fatty acid translocase: after binding to its ligand it facilitates fatty acid transport into cells including myocytes and adipocytes, thereby providing an energy source for these cells. CD36 also exists in a soluble form. Its concentration is fivefold higher in obese diabetic patients than healthy normal-weight patients (Handberg et al., 2006). Macrophages bind CD36 ligands including oxLDL which can trigger secretion of many pro-inflammatory cytokines such as MCP-1/CCL2 and TNF-α. Lipid accumulation in macrophages contributes to foam cell formation (and atherosclerosis progression) and to pro-inflammatory cytokine secretion. CD36 coordinates its action with other signalling partners for example TLRs. CD36 is proposed to mediate crosstalk between adipose tissue macrophages and adipocytes. Mice genetically depleted of CD36 display higher insulin sensitivity and have a lower level of pro-inflammatory cytokines for instance MCP-1/CCL2 and TNF-α (Kennedy et al., 2011).

The data obtained here suggested that CD36 was expressed at a significantly higher level in the IPFP than in synovium in paired sample. There was no difference in CD36 expression by IPFP between the two patient groups (obese vs lean). However, there was a significantly higher expression of CD36 in the synovium of obese OA patients in comparison to lean ones. Barth et al and Rasouli et al found that CD36 gene expression was up-regulated in diabetic subjects (Barth et al., 2010) (Rasouli et al., 2009). Mice with CD36 knockout demonstrated a less inflammatory adipose tissue phenotype and improved insulin sensitivity (Kennedy et al., 2011). Furthermore, subcutaneous and visceral CD36 expression was up-regulated in obesity with no difference between the two fatty tissue depots (Bonen et al., 2006).

HLA-DR (Human Leukocyte Antigen DR) belongs to MHC-II (major histocompatibility complex II). It is expressed by antigen presenting cells (APC), which include macrophages, dendritic cells and others. Its expression in other cell type can be stimulated by IFNγ. Macrophages are the predominant APC in adipose tissue. Diet-induced obesity in mice significantly increases MHC-II expression on gene and protein level and co-localises with $CD4^+$ T cells (Kintscher et al., 2008; Morris et al., 2013). In humans HLA-DR is co-expressed on Adipose Tissue Macrophages (ATM) with CD206 and also co-localises with $CD4^+$ T cells. The same study also showed that mice with genetic depletion of MHC-II were protected from diet-induced obesity, had reduced $CD4^+$ T cells numbers and improved insulin sensitivity (Cho et al., 2014). However, some authors suggest that the majority of cells expressing HLA-DR are adipocytes (Deng et al., 2013). HLA-DR (MHC II) mRNA expression has been reported to be up-regulated in obese subjects (Nair et al., 2005). Adipose tissue macrophages express HLA-DR and the expression of this protein has been shown to be increased in obesity (Bourlier et al., 2008). Mice lacking MHC II have been found to be protected from diet-induced obesity and it has been concluded that MHC II plays an important role in CD4+ T cell activation in obese adipose tissue (Kintscher et al., 2008; Cho et al., 2014). The data presented here suggested that there was an increase in HLA-DR expression in obese subjects in the synovium and IPFP, which may suggest a more pro-inflammatory phenotype of articular adipose tissues in OA in obese patients.

All of the data obtained here suggest that there was a difference in macrophages and macrophage-related markers content in the fatty articular tissues in obese OA patients.

T cells in articular adipose tissue analysis is shown in Table 16:

**Table 15 Summary of T cells analysis in SVF fraction of synovium and IPFP**

| Immune Cell Content Obese (in comparison to Lean) | Synovium | IPFP |
|---|---|---|
| CD3CD4 | ↑ | ↔ |
| CD3CD8 | ↔ | ↔ |
| CD4/CD8 ratio | ↑ | ↔ |

**Legend**
↑ Expressed higher ↔ Expressed at the same level

Analysis of the subsets of T cell populations indicated that there may be differences between lean and obese individuals in these cells content. There was no difference in the total T cell (CD3+) frequency in synovium and IPFP but there was a higher frequency of $CD3^{+}CD4^{+}$ positive cells in the synovium from obese subjects with OA. However, further T cell subpopulation analysis is required as the approach used in this thesis was not sufficient to distinguish T cells subsets including Th1, Th2 or Treg lymphocytes. Tregs, which represent 5-20% of all $CD3^{+}CD4^{+}$ T cells (Cipolletta, 2014) and Th2 (anti-inflammatory) T cell subpopulation (Lee et al., 2014) are for example down-regulated in obesity.

As previously mentioned increased expression of antigen presenting cells (expressing HLA-DR) is reported to induce $CD4^{+}$ lymphocyte influx in visceral adipose tissue

and mediate meta-inflammation (Kintscher et al., 2008; Cho et al., 2014) what concurs with data obtained in this study. Synovial HLA-DR and CD3CD4 cells were increased in obese OA patients. Kintscher et al. also reported that $CD4^+$ T lymphocytes are predominant types of T cells in diet-induced obesity. The ratio of CD4/CD8 was increased in the present study in the synovium of Obese OA patients. However, these differences were not detected in IPFP of the same patient which may suggest that the preliminary pro-inflammatory events may occur mainly in synovium. Although the number of analysed patients in T cells analysis was rather small, therefore in the future these need to be increased.

All obtained data suggest that there are differences in immune cells content in obese articular fatty tissues. These can lead future research into the further characterisation of macrophage and other leukocytes within synovium and IPFP of OA patients in addition to the investigation of their immune role within the knee articular adipose tissue depots.

## 8.3 Subchondral Bone Related Changes

The lateral posterior femoral condyles have been chosen to study the role of obesity on the subchondral bone because of the minimally or no OA-affected subchondral compartment in this region. OA is a disease which is known to correlate with bone sclerosis in such compartments as tibial plateau and medial femoral condyle where cartilage damage and mechanical loading alter the bone microstructure (Li et al., 2013). In the region of choice, no osteoarthritic changes have been detected, therefore all obtained data may be the result of obesity and adipose tissue-derived factors. Furthermore, this is also minimally weight-bearing compartment of the joint which excludes the mechanical role of weight and facilitates the hypothesis of molecular or cellular changes due to obesity.

Calculated bone microstructure parameters (including BV/TV, Tr.Th., Tr.Sp., Tr.N.) on the whole did not show a significant difference between lean and obese patients. The main reason for this may be the low number of samples analysed in this study

(and in future work it may be helpful to increase the sample number). However, after adjustment for the bone mass density (BMD) using hydroxyapatite phantom scan, the data presented here show surprisingly that obese male patients may have a significantly lower bone mass density in comparison to lean ones. The lack of significance in female samples may be due to the postmenopausal/osteoporotic changes. Lean women, although not significantly, were in general older than obese ones. It would be interesting to correlate subchondral bone changes in a higher number of patients with adjustment for age and gender.

Furthermore, a pilot anti-CD68 immunohistochemical staining of subchondral bone indicated that there may be more macrophages within marrow adipose tissue in obese patients with OA. The presence of CD68 positive cells in the marrow of OA patients has been previously characterised (Geurts et al., 2015). My obtained IHC data may suggest that there is an increased number of CD68 positive cells in marrow of obese OA patients. As previously, a higher samples number is necessary in order to draw any conclusion.

The analysis in Chapter 7 has been limited in comparison to previous chapters due to the lack of time. More samples need to be analysed in order to obtain more powerful results. However, these preliminary bone microstructure analysis and osteoblasts gene expression analysis may suggest that the modelling and remodelling processes in the bone of OA patients may be disturbed in association with obesity.

## 8.4 Summary and Future Directions

To sum up, all presented data can contribute to our further understanding of OA. Obtained results may broaden our knowledge about obesity and OA interactions and support the hypothesis that OA in the obese subjects may, in fact, have different origin/pathogenesis than post-traumatic OA. Moreover, the presented study may support the hypothesis that OA in obese subjects may be one of the compartments of the broadly described metabolic syndrome and should be treated/targeted with a different approach than post-traumatic OA. For the future, obesity-related markers (such as adiponectin and PPARγ) are very potent candidates in designing OA modifying drugs. Immune cell-related differences seen in the presented results also suggest different OA phenotype in obese subjects.

Further investigation of the role of adiponectin in cartilage, bone, synovium and IPFP would be beneficial to understand its differential role in all knee joint tissues. It would be interesting to examine the role of two adiponectin receptors and the cascades they are involved in by using such techniques as gene silencing. This could help in explaining reported both catabolic and anabolic role of adiponectin. In the future, it is important to investigate ADIPOR1 and ADIPOR2 protein expression (by Western blot and IHC techniques) in all tissues within knee joint with the special focus on cartilage and subchondral bone to see if obtained gene expression data correlates with protein expression.

Significantly lower PPARγ expression in obese compared to lean osteoarthritic tissues suggests this protein as a very important molecule in OA studies. PPARγ agonists are in common use as anti-diabetic factors. Targeting this nuclear receptor by its agonists (TZDs) or at gene expression level by knock-in techniques (plasmid or viral transgenic methods) may help in modifying OA pathogenesis.

Expression of other obesity-related molecules such as visfatin in OA tissues needs to be further explored by such techniques as mass spectrometry. Visfatin intracellular expression and possible role can be detected with the use of cell fractionation protein

isolation method. That would help in investigating its role as an intracellular enzyme next to the widely reported extracellular adipokine.

Immune cell-related protein (CCL2, TLR4 and VCAM1) higher expression in cartilage (CCL2) and synovium (TLR4 and VCAM1) of obese compared to lean OA patients suggest more proinflammatory phenotype of OA in these patients. Further investigation of the role of the TLR4 receptor by using potent antagonists (such as LPS-RS) could help in examining its role in OA tissues. VCAM-1 mediates immune cell extravasation from the bloodstream. Targeting these events has been previously suggested as a potent anti-inflammatory approach in such diseases as RA or autoimmunity (Yusuf-Makagiansar et al., 2002).

Further characterisation of immune cells content in obese patients synovium and IPFP would be important in defining their role in OA tissues. Subtypes of macrophages and T cells can differentially regulate the immune response in OA obese patients. Thanks to flow cytometry sorting technique it can be also possible to analyse the gene and protein profile of selected cell subtypes (such as $CD206^{+}$ macrophages or $CD3^{+}CD4^{+}$ T cells) and to investigate their possible contribution to OA.

It would be of high interest to conduct a more detailed study on modelling and remodelling processes in subchondral bone in OA in obese patients and to characterise the role of osteoblasts and osteoclasts in this processes. TRAP staining could help to detect osteoclasts in the subchondral compartment of the posterior femoral condyle and define the role of those cells in obesity. Obtained μCT data were not sufficient to conclude if there is a difference between lean and obese women. It would be important in the future to collect a higher number of age-matched samples to analyse the hypothesised difference in obese and lean OA subjects.

To conclude, presented data although do not explain the entire interaction between OA and obesity, adds several novel findings to the knowledge about those two comorbidities.

Appendices

***Appendix* 1**

RNA stability test

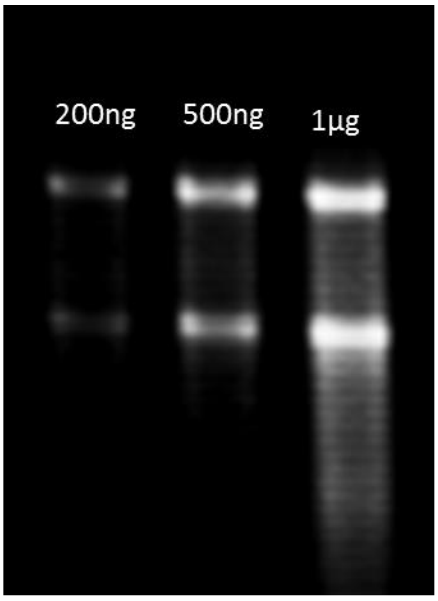



Figure 1 RNA stability test. Synovium 200ng-1ug was loaded on 0.8% agarose gel. Electrophoresis was conducted in MOPS buffer

## *Appendix* 2 Primers for RT-PCR (designed using PRIMER3 software)

| Gene (product size) | *Forward* Primer 5'-3' | *Reverse* Primer 5'-3' |
|---|---|---|
| ACAN(346bp) | TGAGGAGGGCTGGAACAAGTACC | GGAGGTGGTAATTGCAGGGAACA |
| ADIPOQ (228bp) | TGGCTCCACTGGTAAATTCC | TCTCCTTCCCCATACACCTG |
| ADIPOR1 (196bp) | CTTCTACTGCTCCCCACAGC | GACAAAGCCCTCAGCGATAG |
| ADIPOR2 (173bp) | TGGGAAGTTTTGTTCCTTGG | TAGGCCCAAAAACACTCCTG |
| CEBPb (158bp) | GACAAGCACAGCGACGAGTA | AGCTGCTCCACCTTCTTCTG |
| TIG2(229bp) | GAAGAAACCCGAGTGCAAAG | CTT GGA GAA GGC GAA CTG TC |
| CMKLR1 (231bp) | ATCTGGGTC CTG GCTTTCTT | GCAAGCTGTGATGATGAGGA |
| CCL2 (197bp) | CTTCTGTGCCTGCTGCTCAT | CTTGGCCACAATGGTCTTGA |
| COL2A1 (207bp) | AGGAGGCTGGCAGCTGTGTGC | CAGTGGCGAGGTCAG |
| GAPDH (197bp) | GGA GCG AGA TCC CTC CAA AAT | GGC TGT TGT CAT ACT TCT CAT GG |

| | | |
|---|---|---|
| FTO (231bp) | ACCCCTTCACCAAGGAGACT | AAAACTGCAGGCTCAAAGGA |
| LCN2 (167bp) | GTTCACGCTGGGCAACATTA | TCCGAAGTCAGCTCCTTGGT |
| LEP (163bp) | TGCCTTCCAGAAACGTGATCC | CTCTGTGGAGTAGCCTGAAGC |
| LEPR (196bp) | TCCCATATCTGAGCCCAAAG | TGCTTTCACACTGGATGGAG |
| NAMPT (167bp) | GCC AGC AGG GAA TTT TGT TA | GATGTGCTGCTTCCAGTTCA |
| PPARG (225bp) | GCTGTGCAGGAGATCACAGA | GGGCTCCATAAAGTCACCAA |
| TNC (154bp) | CCAAGAAACACCACTTCCTATGTCCTGAG | CCGAATTTTCAGTGATGTCTGAGAAAATGA |
| TLR4 (152bp) | AGCTGTACCGCCTTCTCAGC | CCTGCCAATTGCATCCTGTA |
| VCAM1 (149bp) | TCCTGCTCCGAAAATCCTGT | CTGCTTCTTCCAGCCTGGTT |

***Appendix* 3 Primers for Real Time PCR (designed using PRIMER3 software)**

| Gene (product size) | Forward Primer 5'-3' | Reverse Primer 5'-3' | Efficiency(%) |
|---|---|---|---|
| ADIPOQ (228bp) | GCATTCAGTGTGGGATTGGA | TAAAGCGAATGGGCATGTTG | 96 |
| ADIPOR1 (196bp) | CTTCTACTGCTCCCCACAGC | GACAAAGCCCTCAGCGATAG | 105 |
| ADIPOR2 (173bp) | TGGGAAGTTTTGTTCCTTGG | TAGGCCCAAAAACACTCCTG | 98 |
| B2M (90kb) | TGTGCTCGCGCTACTCTCTC | CCATTCTCTGCTGGATGACG | 100 |
| CCL2 (197bp) | CTTCTGTGCCTGCTGCTCAT | CTTGGCCACAATGGTCTTGA | 95 |
| NAMPT (167bp) | GCCAGCAGGGAATTTTGTTA | GATGTGCTGCTTCCAGTTCA | 105 |
| PPARG (225bp) | GCTGTGCAGGAGATCACAGA | GGGCTCCATAAAGTCACCAA | 100 |
| VCAM-1 (149kb) | TCCTGCTCCGAAAATCCTGT | CTGCTTCTTCCAGCCTGGTT | 104 |

## *Appendix* 4 Anti-Human Primary Antibodies used for IF, IHC and WB technique

| Marker | Species | Type | Dilution in WB | Dilution in IF and IHC | Company |
|---|---|---|---|---|---|
| Adiponectin | Mouse | Monoclonal | 1:500 | 1:100 | Peprotech |
| Visfatin | Rabbit | Polyclonal | 1:10000 | 1:1000 | Peprotech |
| PPARγ | Rabbit | Polyclonal | 1:500 | 1:100 | Santa Cruz Biotech |
| TLR4 | Rabbit | Polyclonal | | 1:100 | BIOSS |
| VCAM1 | Rabbit | Polyclonal | | 1:100 | Peprotech |
| B-actin | Mouse | Polyclonal | 1:1000 | | ABDSEROTEC |
| B-actin | Rabbit | Polyclonal | 1:1000 | | BIOSS |
| GAPDH | Rabbit | Polyclonal | 1:1000 | | SigmaAldrich |

Secondary Antibody

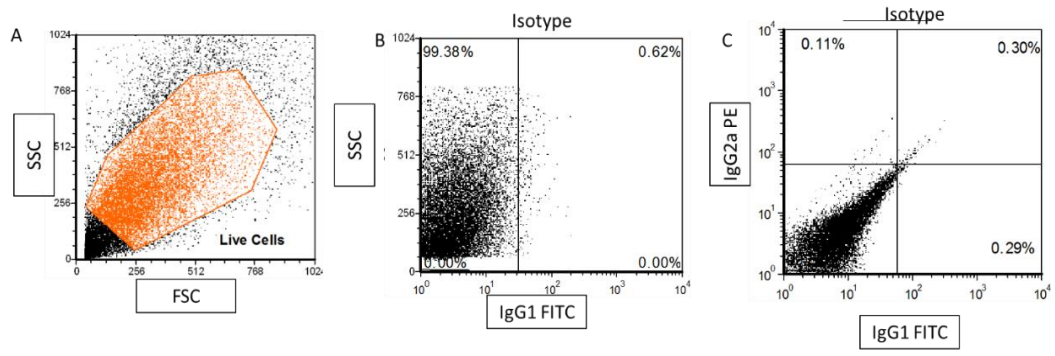
| | Type | Host species | Target species | Conjugate | Dilution in IHC | Dilution in WB | Company |
|---|---|---|---|---|---|---|---|
| IgG (antiserum) | Polyclonal | Rabbit | Mouse | HRP | 1:10000 | | DAKO |
| IgG | Polyclonal | Goat | Rabbit | HRP | 1:10000 | | SigmaAldrich |
| IgG (antiserum) | Polyclonal | Goat | Rabbit | Alexa Fluor 488nm | 1:1000 | | Santa Cruz Biotech |
| IgG | Polyclonal | Goat | Mouse | IRDYE680RD | | 1:10000 | LiCOR |
| IgG | Polyclonal | Goat | Rabbit | IRDYE800CW | | 1:10000 | LiCOR |

Antibodies for Flow Cytometry

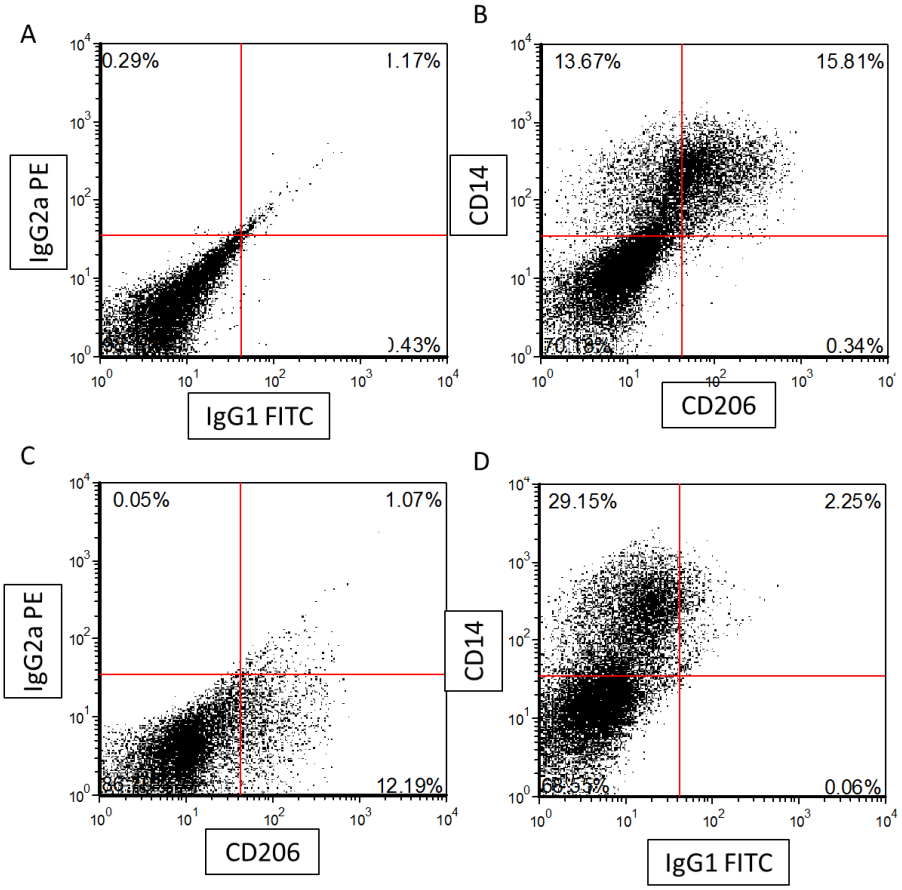
| Marker | Isotype | Conjugate | Species | Dilution | Company |
|---|---|---|---|---|---|
| VCAM1 | IgG1 | PE | Mouse | 1:100 | BioLegend |
| TLR4 | IgG1 | PE | Mouse | 1:100 | BioLegend |
| CD45 | IgG1 | FITC | Mouse | 1:100 | ImmunoTool |
| CD14 | IgG1 | PE | Mouse | 1:100 | ImmunoTool |
| CD206 | IgG1 | Alexa Fluor | Mouse | 1:100 | BioLegend |
| CD86 | IgG1 | FITC | Mouse | 1:100 | ImmunoTool |
| CD36 | IgG1 | FITC | Mouse | 1:100 | ImmunoTool |
| HLA-DR | IgG2a | FITC | Mouse | 1:100 | ImmunoTool |
| CD11b | IgG2a | PE | Mouse | 1:100 | ImmunoTool |
| CD18 | IgG1 | PE | Mouse | 1:100 | ImmunoTool |
| CD3 | IgG1 | FITC | Mouse | 1:100 | ImmunoTool |
| CD4 | IgG2a | PE | Mouse | 1:100 | ImmunoTool |
| CD8 | IgG2a | PE/Dy647 | Mouse | 1:100 | ImmunoTool |
| control | IgG2a | PE | Mouse | 1:100 | ImmunoTool |
| control | IgG2a | FITC | Mouse | 1:100 | ImmunoTool |
| control | IgG1 | PE | Mouse | 1:100 | ImmunoTool |
| control | IgG1 | FITC | Mouse | 1:100 | ImmunoTool |
| control | IgG2a | PE/Dy647 | Mouse | 1:100 | ImmunoTool |

**Appendix 5**

Flow Cytometry Gating Strategy for SVF from IPFP and synovium analysis.



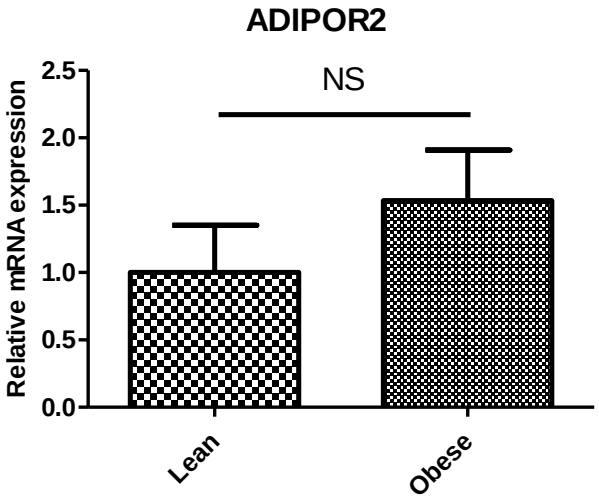

Gating strategy shown in A. Isotype control staining for single (B) and double marker staining (C) example shown.



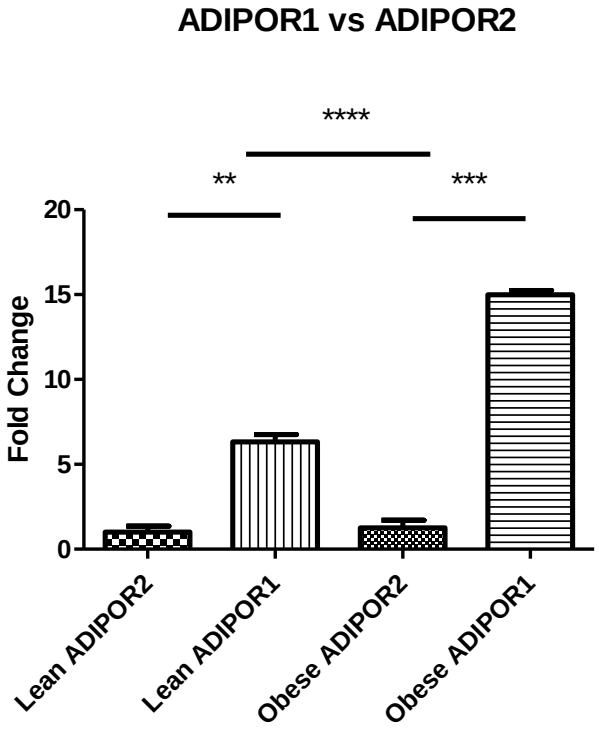

Example of double colour staining (B) for CD14CD206 cells in SVF. Single colour staining shown in (C,D). Isotype control shown in A.

**Appendix 6**

ADIPOR2 expression in chondrocytes by Real Time PCR analysis




Difference between ADIPOR1 and ADIPOR2 in chondrocytes assessed by Real Time PCR



**Appendix 7**

IgG controls for IF and IHC techniques

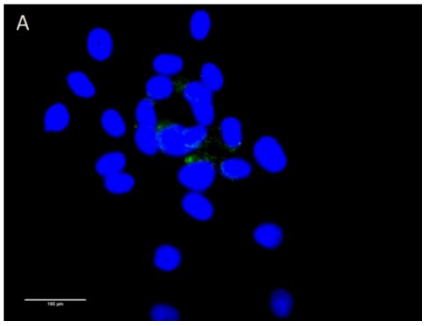



Figure 1 Isotype control staining for chondrocyte IF analysis. Isotype non-specific rabbit IgG plus secondary goat anti Rabbit IGG conjugated with FITC was used as described in Material and Methods.

A B

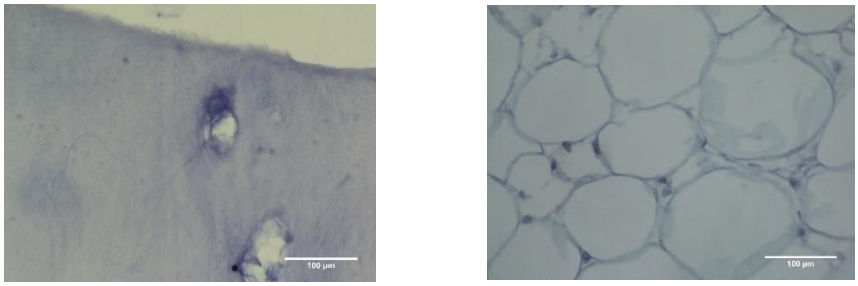


Figure 2 Isotype control staining for cartilage IHC and synovium analysis. Isotype non-specific rabbit IgG plus secondary goat anti Rabbit IgG conjugated with HRP was used as described in Material and Methods.

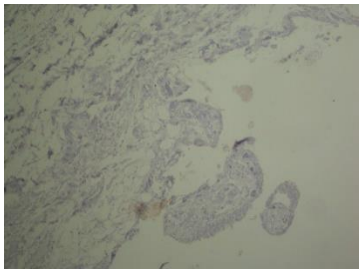


Figure 3 Isotype control staining for synovium IHC analysis. Isotype non-specific mouse IgG plus secondary Rabbit anti-mouse IgG conjugated with HRP was used as described in Material and Methods.

## Appendix 8

## Synovial and IPFP Histology

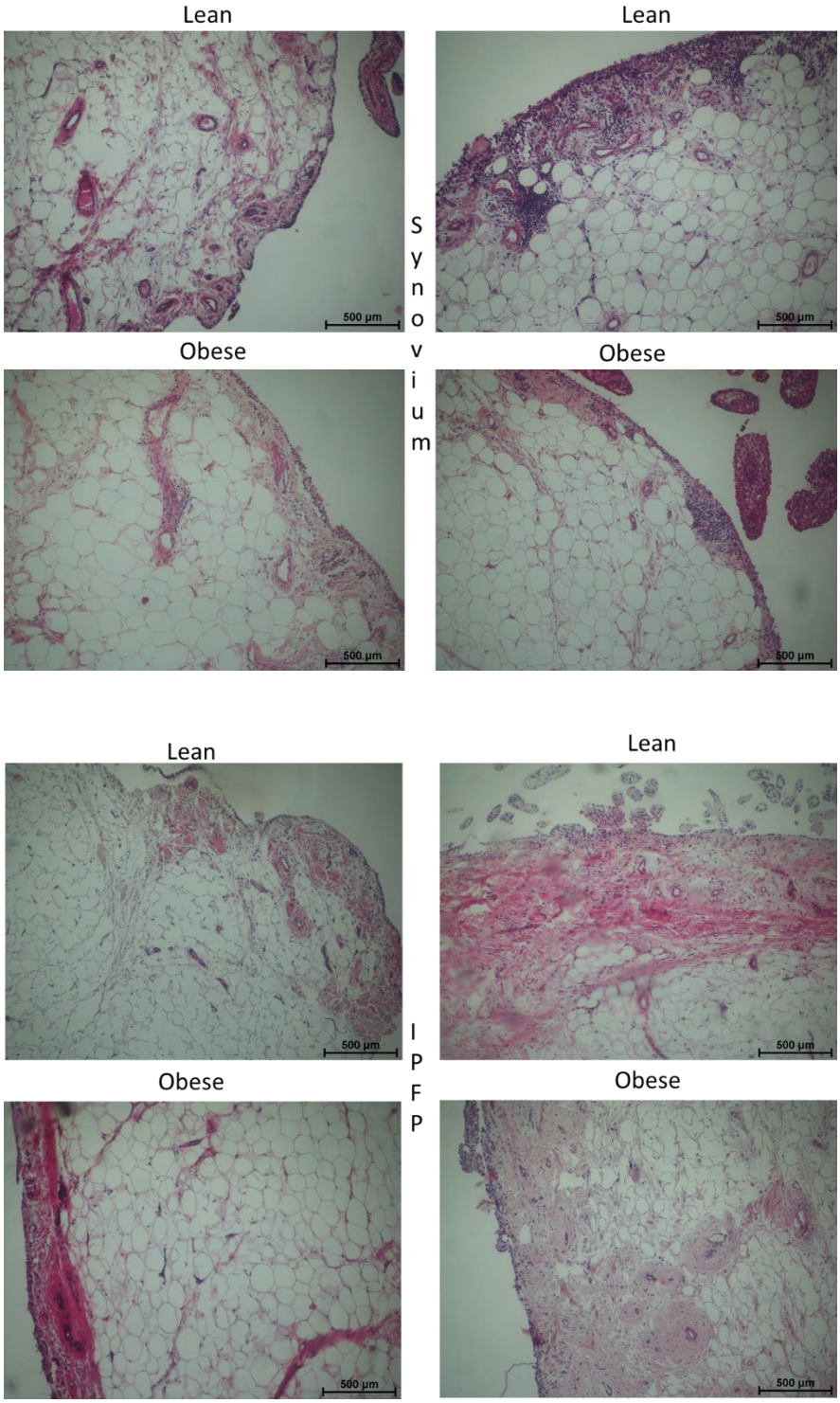
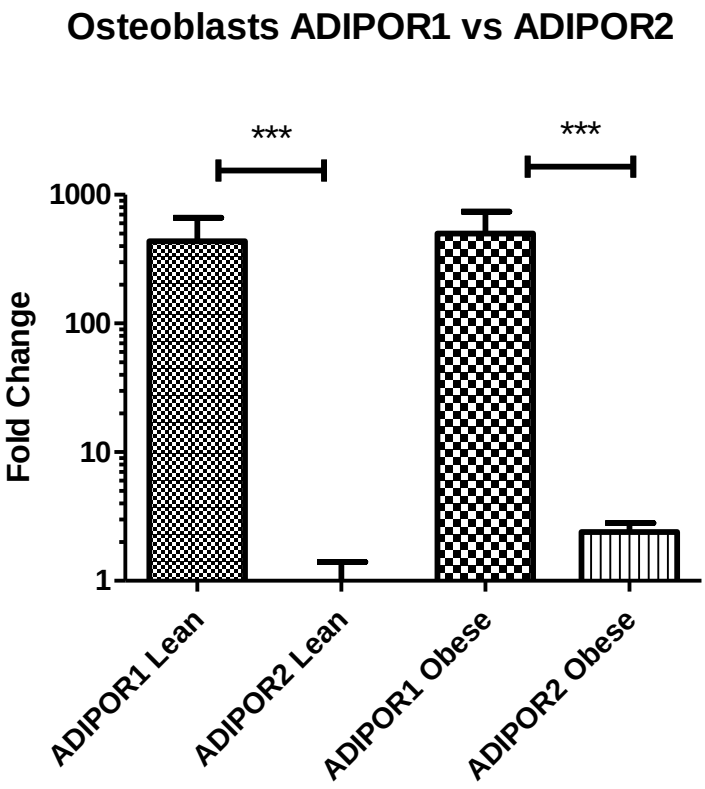


Figure 4 Representative Hematoxylin and Eosin (H&E) staining for Synovial and IPFP biopsies.

***Appendix* 9 Osteoblasts ADIPOR1 and ADIPOR2 gene expression analysis**



**Appendix 10**

Poster Presentation

September 2013

Edinburgh Pathology 2013 meeting Poster Presentation on “Osteoarthritis (OA) and Obesity- Are the Co-Culprits?”

September 2013

British Orthopaedic Research Society meeting. Poster presentation on “Are Osteoarthritis and Obesity Accomplices”

National (UK) Conference oral presentation

September 2015

British Orthopaedic Research Society meeting. Podium presentation on “Are adipose tissue macrophages potent agents in the pathogenesis of OA?”

July 2014

British Orthopaedic Research Society meeting. Podium presentation on “The role of adipokines in End-Stage knee Osteoarthritis”

International Conference Oral Presentation

March 2016

Orthopaedic Research Society meeting. Podium presentation in Spotlight Session on “Adipose tissue macrophages at the Crossroad between Obesity and Osteoarthritis”

Awards

ImmunoTools-Award 2012 for the project on “The Role of Obesity in Osteoarthritis”

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